HUMAN PAPILLOMAVIRUS : PATHOGENESIS AND IMMUNITY

HEATHER A. CUBIE, BSc., M.Sc.

Thesis submitted for the Degree of Doctor of Philosophy

University of Edinburgh

1989

DECLARATION

The investigations and procedures described herein were performed by me and the composition of this thesis is entirely my own.

Signature

"For the absurd man it is not a matter of explaining and solving, but of experiencing and describing. Everything begins with lucid indifference".

A. Camus in "The Myth of Sisyphus"

"Interaction of viral infected cells with the immune system is not a simple event ..... Only the detailed understanding of the strategy of survival of the individual viruses and the pathogenesis of the diseases they produce will enable one to predict the consequences of stimulating the immune response to all or any of their antigens".

P.J. Lachman (1985) Brit Med Bull 41 p3

"Warts are wonderful structures ..... The strongest thing about warts is that they tend to go away ..... Some intelligence or other knows how to get rid of warts ..... It is a wonderful problem, in need of solving ..... It would be worth a War on Warts, a Conquest of Warts, a National Institute of Warts and All".

Lewis Thomas, "On Warts" in "The Medusa and the Snail", Notes of a Biology Watcher; The Viking Press, New York.

DEDICATION

This thesis is dedicated to the memory of two people who died within a few months of each other and who would have been pleased to see its completion : firstly to my Father, William McDonald Muir, who died on 16th August, 1986, who added the stress of my endeavours to his own, and secondly to Elizabeth Edmond, who died on 8th December, 1986, friend of long-standing, mentor and colleague, whose endurance and courage in the face of great difficulties will continue to stimulate me.

ABSTRACT

The involvement of human papilloma virus (HPV) in the aetiology and progression of cervical intraepithelial neoplasia (CIN) is still unresolved. This study was designed to assess the immunological responses to HPV types in patients presenting with varying degrees of CIN and in control groups, using in vitro measures of cellular and humoral responses.

Lymphocyte proliferation assays (LPA) were performed using peripheral blood mononuclear cells (PBM) and various papillomavirus (PV) antigens. Twenty-five per cent (23/92) of patients with CIN responded to antigens derived from purified BPV, HPV-1 or HPV-2 with or without detergent disruption. The responses correlated with a past history of skin warts rather than cervical abnormalities, and the percentage of responders was similar to that in laboratory personnel (30%) and lower than that in a group with recalcitrant common warts (50%). Antigens specific to HPV-16 and HPV-18, in the form of bacterially expressed fusion proteins derived by the transcription and translation of the E6 and E4 open reading frames (ORF), occasionally produced specific positive responses, provided contaminating E.coli B galactosidase sequences had been removed during purification. Responses were low and suggested that the numbers of memory T cells specific to PV antigens were low and at the lower limit of detection of LPA. An indirect ELISA was developed to detect circulating IgG to PV antigens in colposcopy patients. Fifty per cent of patients had antibodies to disrupted HPV-1, HPV-2 or both, suggesting that a predominantly type-specific response was being detected. No correlation of immune responses with a degree of dysplasia or the presence of koilocytes in cervical biopsies was noted, but a high incidence of forgotten or inapparent past infection with cutaneous HPV types was found.

In situ hybridisation (ISH) methods using non-radioactively labelled, cloned probes and synthetic oligonucleotide probes were developed for use on paraffin sections. Synthetic probes allowed a quicker, less destructive hybridisation protocol, with the sensitivity of detection being

improved by an anti-biotin-immunogold conjugated immunoglobulins-silver enhancement (IGSS) detection system. Double staining of PV antigen and nucleic acid on the same section was achieved. Synthetic oligonucleotides offer an exciting new tool for diagnostic virology, worthy of exploitation in many systems.

Implantation of human foreskin infected with HPV-11 was shown to provide an animal model, albeit technically difficult, in which HPV could be produced, but a more practical technique of productive HPV infection in vitro is still required if the biology and pathogenesis of HPV infections is to be clarified further.

TABLE OF CONTENTS

(Major headings only; subheadings detailed under each section of manuscript)

LIST OF ILLUSTRATIONS

LIST OF TABLES

INTRODUCTION

INTRODUCTION

INTRODUCTION

1. General Introduction

Papillomaviruses form one genus of the Papovaviridae family of small icosahedral viruses containing double-stranded circularly closed DNA. Papillomaviruses (PV; from papilla meaning nipple and oma meaning tumour) are widely distributed in nature and occur in numerous animal species including rabbits, hamsters, sheep, goats, deer, cattle, horses, dogs, monkeys and even birds (Pfister,1984). Man is no exception and human papillomavirus (HPV) has long been recognised as the causative agent of skin warts (Strauss et al., 1950). As such it was considered to be the first known human tumour virus (Rowson & Mahy, 1967). The structural properties of the virus were well worked out in the early 1960's because large quantities of virus could be obtained from clinical lesions such as plantar warts, but, in defying all attempts to culture it in vitro, HPV effectively by-passed that era of virology when the biology and pathology of viral infectivity and replication was worked out for those viruses which grew readily in cell culture. The literature of the 60's and early 70's is full of unsuccessful attempts to learn more of the infectious nature of this virus (see Cubie, 1972a). It required, however, the leap into biology at the molecular level rather than virology at the particle level for progress to be achieved and interest in HPV to be renewed.

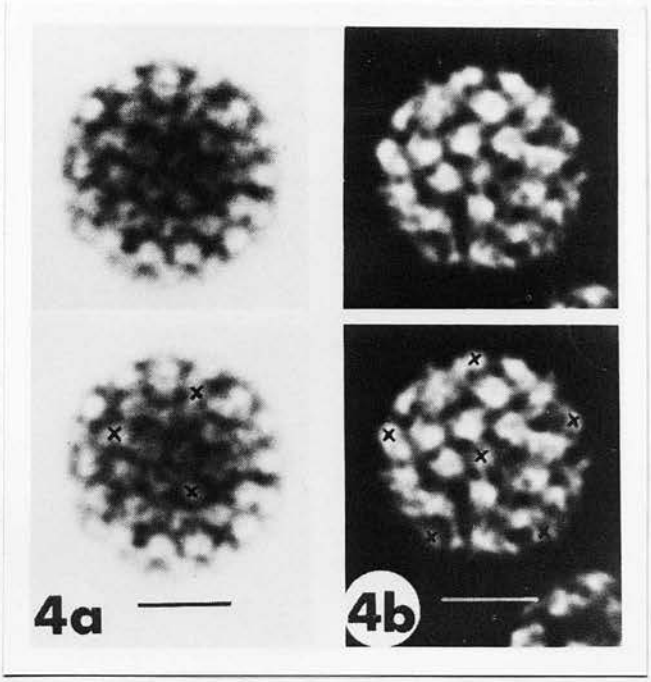
The last ten years have seen a fruitful collaboration between molecular biologists, virologists, clinicians and veterinarians, and the picture of HPV involvement in tumour progression which has unfolded is fascinating and still far from complete. The work presented here offers a further fragment in the enigmatic jigsaw of HPV pathogenesis.

2. General Characteristics of Papillomaviruses

2.1. Virus Structure

The Papillomaviruses (PVs) are non-enveloped, icosahedral particles of 50-55 nm in diameter (Crawford and Crawford, 1963) with the capsid composed of 72 capsomeres, and for human papillomaviruses these are arranged in a righthanded lattice (Klug and Finch, 1965). The subunits have a hollow, cylindrical structure (Noyes, 1964) and are connected at their base by bridge-like structures (Yabe et al., 1979) as shown in Figure 1. Double stranded DNA is contained within the capsid and "full particles" can be readily separated by equilibrium gradient centrifugation in caesium chloride from "empty particles" devoid of DNA (Breedis et al., 1962).

Virion protein makes up about 88% of the particle mass (Crawford, 1969) with up to ten polypeptides having been detected in different PVs on polyacrylamide gel electrophoresis (PAGE) after sodium dodecyl sulphate (SDS) disruption (Favre et al., 1975a). The capsomere subunit in each PV type was represented by the major polypeptide of MW 54,000 daltons and in different studies there appeared to be up to six irregularly detected polypeptides (Favre et al., 1977; Pfister et al., 1977; Lancaster and Olson, 1978). These were considered to be breakdown products of the major polypeptide, produced by the use of trypsin during purification (Pfister et al., 1977). In addition, four low molecular weight polypeptides closely associated with DNA and comparable to cellular histones were resolved (Lancaster and Olson, 1982). Using further purification procedures, Meinke and Meinke (1981) confirmed the major capsid protein to have a MW of 53,500 daltons, to be very acidic and negatively charged as with structural subunits of other viruses. These workers also noted two slightly smaller polypeptides, the histone-like peptides and in addition, two large polypeptides in low concentration but with MWs greater than 70,000 daltons. The inability to obtain viral material free of contaminating



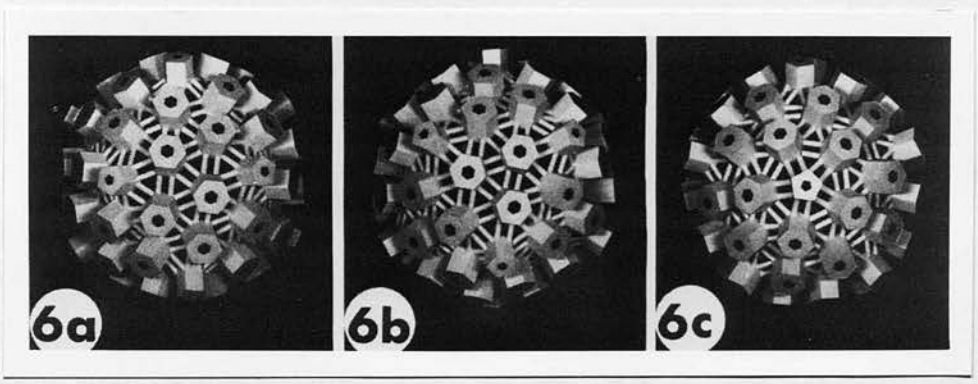


Fig 1 Structure of HPV Particles

A. Negatively stained particles showing hexagonal (a) and pentagonal (b) contours.

B. Tentative model showing twofold (a), threefold (b) and fivefold (c) axes of symmetry.

(From Yabe Y, Sadakane H and Isono H (1979): Virology 96, 547-552).

protein made these early results difficult to interpret, and it is only very recently, by the use of molecularly cloned and expressed viral fragments that more light has been shed on the protein content of PVs (vide infra).

The DNA of all PVs is double stranded, circular and supercoiled (Crawford, 1969) and has a MW of 5×10^6, corresponding to approximately 8000 base pairs (bp) (Kleinschmidt et al., 1965). Experiments on polyoma using mild chemical reducing agents to induce progressive scission in the chain (Vinograd et al., 1965) suggested the unusual twisted circular form for papovavirus DNA and, in electron micrographs of HPV, supercoiled (Component I) and open rings (Component II) are easily seen (Follett and Crawford, 1967). There are 20 turns in the supercoiled form, estimated by the use of ethidium bromide as an intercalating agent (Crawford and Waring, 1967).

The DNA of the cottontail rabbit papilloma virus (CRPV), described by Shope in 1933, was the first tumour virus DNA to be analysed in detail, and was found to have an adenine plus thymine (A+T) to guanine plus cytosine (G+C) ratio of 1.08 (Watson and Littlefield, 1960). Buoyant density measurements of HPV-DNA showed a slightly lower G+C content (Crawford and Crawford, 1963). Nevertheless, using the current technology of the time with nearest neighbour sequence analysis, Subak-Sharpe and his colleagues (1966) concluded that HPV and CRPV were similar to each other and to their host DNAs in the patterns of doublets obtained.

2.2 Papillomavirus Plurality and Relatedness

2.21 Early Studies

The infectious nature of skin warts and their transmissibility within sterile cell free filtrates of lesions was well recognised in the later 19th and early 20th centuries (Ciuffo, 1907 and see Cubie, 1972a). Not long after, the apparent identity between the filterable agent of

genital warts and common warts was shown by transmission experiments (Waelsch, 1918) and for more than 50 years thereafter, despite the diversity of clinical lesions induced, it was thought that the same agent was involved. Differences in clinical morphology were thought to be related to anatomical site, the age of the lesion and to host-related factors (Rowson and Mahy, 1967). This belief was maintained by early studies searching for antigenic variation: reactions of identity in Ouchterlony gel diffusion tests with human antisera were obtained using antigens from simple plantar warts, mosaic plantar warts and common hand warts (Ogilvie, 1970b). On the other hand, Almeida and her colleagues in 1969 suggested that antigenic differences did exist between skin wart virus and the virus causing genital warts with a one way serological cross between them : antisera to skin warts reacted with both skin and genital wart viruses while antisera to genital warts reacted only with genital warts. Epidemiological data suggesting that venereal transmission was the main route of infection with genital warts (Oriel, 1971a) added weight to the view that two HPV types existed and the absence of sequences homologous to skin wart DNA in genital warts in the first molecular hybridisation experiments (zur Hausen et al.,1974; Delap et al., 1976) supported this.

Early on it had also appeared that there was no antigenic cross-reaction between papilloma virions of different species in immunodiffusion studies (Le Bouvier et al., 1966); Favre et al., 1974). However, common antigenic determinants could be detected in plantar wart sections by using serum from rabbits bearing CRPV-induced carcinomas in which virus replication was incomplete (Orth et al., 1978). The effect could be neutralised by preincubation with alkali disrupted HPV virions but not with intact particles. These results suggested the existence of a common internal antigen and this was confirmed by Jenson et al., (1980) using antiserum to SDS-disrupted BPV.

2.22 Molecular Advances

Following the discovery of the specific nature of the double stranded breaks produced by restriction endonucleases (Smith and Wilcox, 1970), together with the ability to separate and resolve the resulting fragments by electrophoresis, rapid advances were made in the study of the structure of the genomes of PVs. The first physical map of HPV-DNA was produced by Favre et al. (1975b) using DNA from plantar warts. In addition to the three breaks produced by HindII and HindIII endonucleases, they found two Eco RI cleavage sites in their preparation. However, the observation of different but very weak electrophoretic bands at the detection limit of their system, suggested to these authors the presence of sequence rearrangements, or, as became increasingly and rapidly apparent, HPV "variants". In 1974, zur Hausen and colleagues had shown that cRNA to component I DNA from plantar warts did not hybridise with all common wart samples tested, and in 1976, Gissmann and zur Hausen found two different cleavage patterns in three individual preparations of plantar warts. By 1977, the same workers had described four different cleavage patterns from 26 separate preparations of plantar and common warts, and called them HPV-1, 2, 3, and 4 (Gissmann et al., 1977). Orth's group in Paris (Orth et al., 1977) similarly described three types, one of which corresponded to the most frequently found pattern in Gissmann's study, and which was associated with plantar warts rich in virus particles. These preparations were also designated HPV-1, 2 and 3. At the same time, two classes of bovine papilloma virus (BPV-1 and BPV-2) were described by Lancaster & Olson (1978), and it became clear that a classification system for PVs was required. Following an international meeting in Alabama in 1978, a scheme was established (Coggin & zur Hausen, 1979) whereby papillomaviruses would be designated PV and the species conveyed by the first two letters of the specific name, with the exception of human and bovine PVs which were designated by convention as HPV and BPV. New preparations could be considered independent

types when there was less than 50% homology between them as shown by DNA-DNA hybridisation and sub-types where the homology was incomplete but >50%. Where possible, type-specific differences were to be confirmed by significant titre changes in reciprocal assays of standard serological procedures.

A major problem, however, in these early studies on the DNA isolated from individual lesions, was the minute amount of nucleic acid available for such tests. The use of molecular cloning of PV genomes into bacterial plasmid, (Heilman et al., 1980 ; De Villiers et al., 1981), however, enabled large quantities of homogeneous genetic material to be produced and further increased the intensity of the search for additional HPV types. By 1987, 50 types of HPV had been differentiated (Pfister, 1987a) and in the last year a further 7 have been accepted as new types (von Knebel Doeberitz et al., 1988). The identification of an unknown papilloma virus is currently based on molecular cloning of its DNA followed by blot hybridisation to all known types (Gissmann, 1984). These types are organised in groups according to the extent of their homologies and these are shown in Table 1, with the common types depicted in bold. Only a few types have been totally sequenced, in particular, HPV-1a (Danos et al., 1982); HPV-6b (Schwarz et al., 1983); HPV-16 (Seedorf et al. 1985 and corrected by Baker et al., 1987); HPV-11 (Dartman et al., 1986); HPV-18 (Cole & Danos., 1987) and HPV-5 (Zachow et al., 1987). Because of its heterogeneity, HPV-2 remains unsequenced. The judicious use of single restriction endonucleases allows differentiation of many HPV types and this is shown in Figure 2 for the enzyme Pst 1.

While many more types have been recognised in the human system, there are now known to be at least six types of BPV of which BPV-1, BPV-2 and BPV-4 have been sequenced (Chen et al., 1982; Groff, Mitra and Lancaster, 1986; Patel et al., 1987, respectively). So far only a single type of CRPV has been recognised and the sequence was determined by Giri et al., (1985).

Table 1. Human papillomavirus types grouped according to DNA sequence homology (adapted from Pfister, 1987)

Group	A	B	C	D			E	F	G	H	I	J	K	L	M	N	O	P	Q	Unclassified
	1	2	4	5	9	24	6	7	16	18	26	30	33	34	35	39	41	43	50	48, 53-55
		27		8	15		11		31	32	51*		52+							
		29		12	17															
				14	37		13			42										
		3		19	38		44			45										
		10		20																
		28		21																
				22																
				23																
				25																
				36																
				46																
				47																
				49																

* Nuovo et al., 1988

\+ Noda et al., 1988

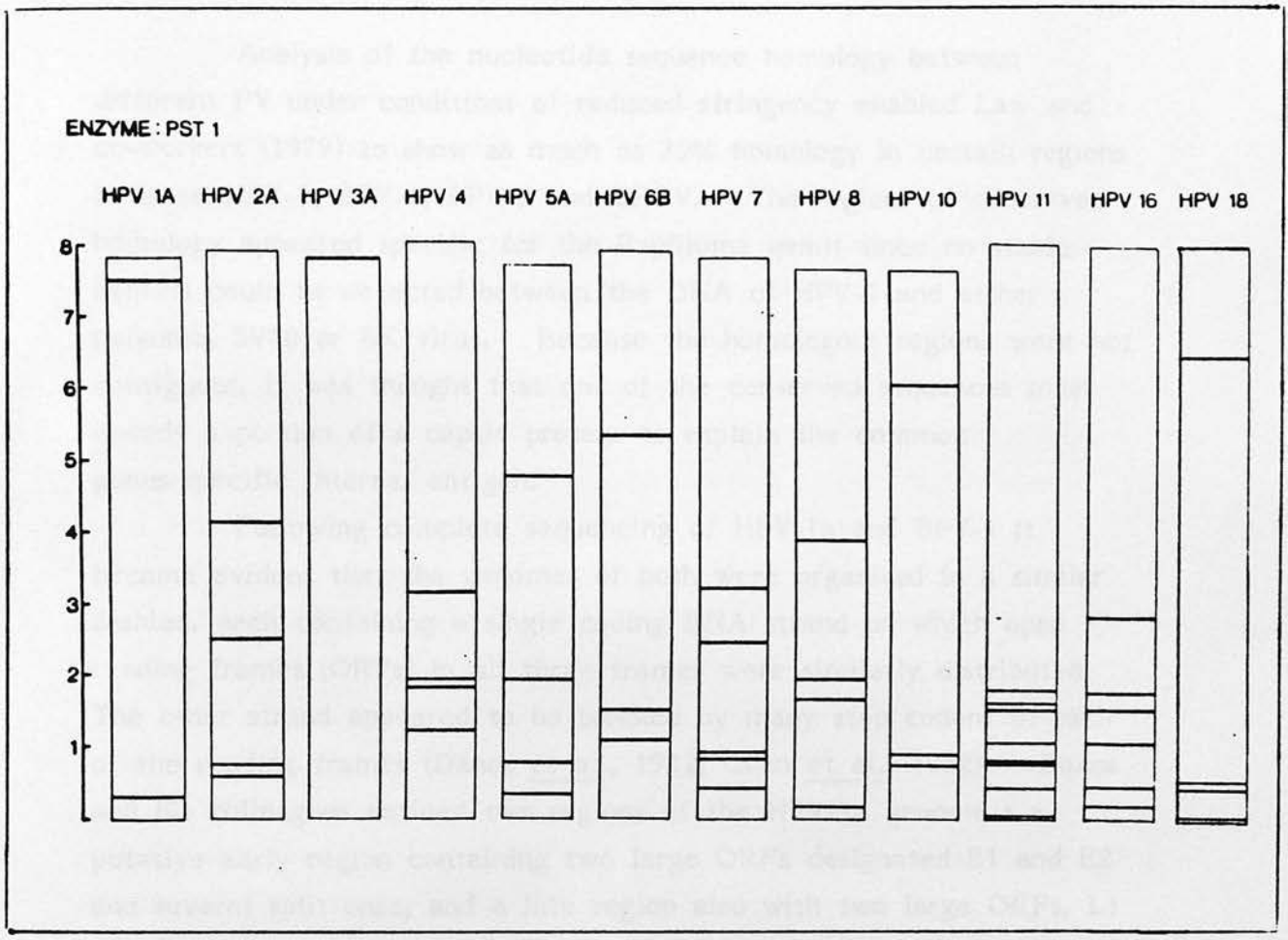


Fig 2. Pst-1 restriction site maps of common HPVs (adapted from von Knebel Doeberitz C, Bunge R, Buchert J and de Villiers E-M, 1988).

2.3 Genetic Organisation in Papillomaviruses

Analysis of the nucleotide sequence homology between different PV under conditions of reduced stringency enabled Law and co-workers (1979) to show as much as 70% homology in certain regions between HPV-1, BPV-1, BPV-2 and CRPV. The regions of conserved homology appeared specific for the Papilloma genus since no stable hybrids could be detected between the DNA of HPV-1 and either polyoma, SV40 or BK virus. Because the homologous regions were not contiguous, it was thought that one of the conserved sequences must encode a portion of a capsid protein to explain the common genus-specific internal antigen.

Following complete sequencing of HPV-1a and BPV-1 it became evident that the genomes of both were organised in a similar fashion, each containing a single coding DNA strand on which open reading frames (ORFs) in all three frames were similarly distributed. The other strand appeared to be blocked by many stop codons in each of the reading frames (Danos et al., 1982; Chen et al., 1982). Danos and his colleagues defined two regions of the HPV-1a genome : a putative early region containing two large ORFs designated E1 and E2 and several split ones, and a late region also with two large ORFs, L1 and L2, considered to encode the structural polypeptides (Danos et al., 1982). In 1980 an American group had shown that both BPV DNA from virons, and cloned BPV-1 or BPV-2 DNA could transform mouse cells (Lowy et al., 1980). A cloned fragment representing 69% of the genome was all that was required and five distinct transcripts mapping on this fragment were found within transformed cells. The 31% fragment was not expressed in the transformed cells but only in productively infected cells. This fragment, like the L region of HPV-1a, was shown to contain two large ORFs separated by a single translational stop codon and to encode the major capsid protein (Chen et al., 1982). By working together, the French and American workers showed most elegantly how similar was the overall organisation between

HPV-1a and BPV-1. By aligning the regions of greatest homology (in L1, E1/E2 and the beginning of L2), and starting the nucleotide numbering from the Hpa-I site in both, eight ORFs coding for peptides of more than 90 amino acids were designated in the early region and numbered E1 to E8 on the basis of their size, homology and location, while two ORFs encoding structural proteins, designated L1 and L2 and transcribed only in productively infected cells were found in the late region (Danos et al., 1983). The L1 of both viruses showed extensive homology, with divergence occurring simply by an accumulation of point mutations. The L2 regions were much more variable and it was suggested that the peptide coded by this region might play a role in host restriction.

Little homology was detected in the region of the genomes where E2, E3 and E4 overlapped. Polypeptides encoded by E6 and E7 would have sparse amino acid homology but would be rich in Cys-X-X-Cys moieties suggesting that the tertiary structure would be maintained despite divergence in the primary amino acid sequence. E5 and E8 were found to be quite different on each viral genome. Finally, a non-coding region (NCR) between the end of L1 and start of E6 also showed several conserved sequences, suggesting regulatory signals for viral gene expression and DNA replication. Recently it has been shown that the NCR contains not only promoter and enhancer elements, but also a glucocorticosteroid-reactive element (GRE) which can be activated by dexamethasone with resulting increase in transcription (Gloss et al., 1987).

This important comparative analysis led the way for further studies on the genomic organisation of different papillomaviruses, and despite considerable sequence heterology, similar organisation was shown in the first ten papillomaviruses whose complete DNA sequences were determined (Giri and Danos, 1986). A composite figure of several established profiles is presented in Figure 3. Nevertheless, it must be remembered that overall sequence homology may not be enough to give

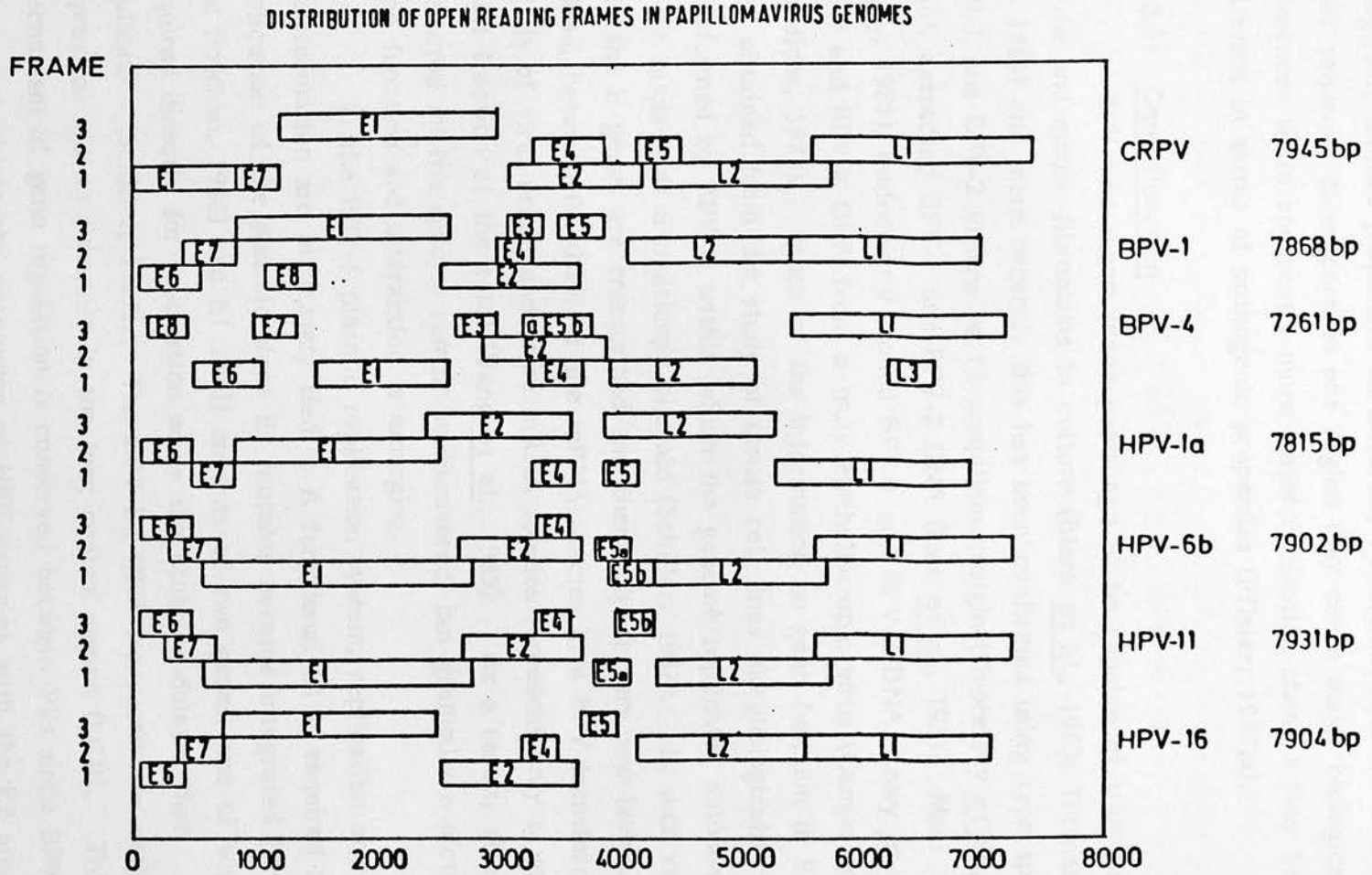


Fig 3. Genomic organisation of CRPV, BPV-1, BPV-4, HPV-1a, HPV-6b, HPV-11 and HPV-16.

biologically similar properties to different Papillomaviruses. Indeed, minor sequence divergence in one region may cause major biological differences while apparently more major nucleotide changes may be irrelevant in terms of pathogenic properties (Pfister, 1987a).

2.31 Gene Function

BPV was shown many years ago to be capable of transforming bovine and mouse fibroblasts in culture (Black et al., 1963; Thomas et al., 1964) and more recently this has been confirmed using type specific BPV-1 and BPV-2 virions of fibropapilloma origin (Dvoretsky et al., 1980), extracted BPV-1 and BPV-2 DNA (Law et al., 1981; Moar et al., 1981), molecularly cloned BPV-1 and BPV-2 DNA (Lowy et al., 1980) and BPV-4 DNA from a truly epitheliotropic virus (Campo and Spandidos, 1983). Much of the information on gene function in PV has been obtained from the study of mouse cell lines morphologically transformed by BPV-1, within which the genome replicates autonomously in the nucleus as a multicopy plasmid (Schiller, 1987). In such cells, only the E genes are transcribed and then only at very low levels. Indeed, between 0.1-0.2% of the mRNA species in a BPV transformed cell is of viral origin and some mRNA species represent only a very small fraction of the total (Yang et al., 1985). As a result, several predicted mRNA species remain undiscovered, but gradually a picture of gene function and interaction is emerging.

In the BPV-1 plasmid replication system, replication and transformation are intricately tied. A functional E1 is required for replication of the plasmid since E1 mutants become integrated (Broker and Botchan, 1986) and E1 itself consists of two genes, one of which is required directly for replication while the other modulates that replication (Schiller, 1987). The BPV E2 ORF transactivates early gene expression via an enhancer in the long control region (LCR). This mechanism of gene regulation is conserved between PVs since BPV E2 can transactivate the expression of HPV genomes with the E2 product

binding directly to the conserved enhancer sequence ACCG-CGGT (Phelps and Howley, 1987). Very recent extension of these findings has shown that different E2 proteins transactivate transcription by a common mechanism (Giri and Yanif, 1988). Not only did the E2 of HPV-18 or BPV-1 transactivate the LCR of CRPV, but the E2 of CRPV acted similarly on the LCR of BPV-1, HPV-1 and HPV-18.

The E6 gene is capable of independently transforming C127 mouse cells (Schiller et al., 1984). It can also act synergistically with E5, another independent transforming gene, which is so similar in those PVs which induce fibropapillomas that it may play a major role in the proliferation of dermal elements (Schiller, 1987). E7 does not have intrinsic transforming ability, but in the BPV-1 system it can act with E6 to play a role in the maintenance of high plasmid copy number (Lusky and Botchan, 1985).

It is becoming apparent, however, that BPV gene functions cannot be extrapolated entirely to the HPV system. In HPV-16 and HPV-18, for example, transforming activity has been principally assigned to E7 (Smotkin and Wettstein, 1987; Kanda et al., 1988), and E5 is often deleted during integration of HPV-16/18 into the host genome (Schwarz et al., 1985). The E6-E7 region is consistently transcribed in cervical tumour cell lines with three transcripts, E6, E7 and E6* formed by the removal of 182 nucleotides from within E6 (Schwarz and Schneider- Gadicke, 1986). Sequence comparison suggests the spliced transcript could only be found in HPV types 16, 18, 31 and 33 and not in HPV-1, or HPV-6/11 which lack the appropriate donor-acceptor splice sites (Schwarz et al., 1987). Thus the E6* transcript may provide an important difference between high and low oncogenicity in HPV types. *

Details of the currently established functions of BPV genes are presented in Table 2, together with known functions of HPV and CRPV genes.

Additional gene functions for particular sequences have been suggested. In 1984, Clertant and Seif noted that the putative E1

Table 2. Gene functions in PVs with particular reference to the BPV-1 transforming system, but including known functions of HPV and CRPV

ORF	Approx. AA coding capacity	Function of BPV-1 system	Reference	Function in other PV systems	Reference
E1	600	Episomal Maintenance - 2 genes 'R' and 'M' acting as positive and negative replication modulators	Lusky & Botchan 1984 Schiller, 1987	HPV-16 E1/E2 may have tumour suppressing activity	Yutsudo et al., 1988
E2	300-400	Transactivation of early gene expression Antagonistic transcription repression transcriptional repressors	Spalholz et al., 1985 Yang et al., 1985 Haugen et al., 1987	Transactivation of transcription in HPV and CRPV by common mechanism	Phelps & Howley, 1987 Giri & Yaniv, 1988
E3		Unknown			
E4	100-200	Appears to have no function in early events	Neary et al., 1987	In HPV-1 may be involved in virion maturation or keratin disorganisation	Doorbar et al., 1986 Breitburd et al., 1987
E5	50	Independent transforming gene, preferentially involved in induction of dermal elements of fibropapillomas	Schiegel et al., 1986 Schiller et al., 1986	HPV 5/8 lack E5 Only HPV6 has E5 closely related to that of BPV. In human cervical carcinomas 3' end of early region which would contain E5 (and E1,E2) selectively lost or interrupted. In HPV-16 extra 'T' in sequence gives 83AA, transmembrane protein which induces cell proliferation	Iftner et al., 1988 Schwarz et al., 1985 Bubb et al., 1988
E6	140	Independent transforming gene, acts synergistically with E5	Yang et al., 1985	HPV-18 E6/E7 transforming function, possibly more efficient than whole genome	Bedell et al., 1987
E7	100	Maintenance of high copy number Maintenance of transformed state and differentiation	Lusky & Botchan, 1985	Transforming gene in HPV-16/18	Smotkin & Wettstein, 1986; 1987 Kanda et al., 1988
E8	100	Unknown			
L1	500	Encodes major capsid protein and shows very little variation	Engel et al., 1983	Encodes major capsid protein in all other PVs examined	
L2	500	Encodes minor capsid protein, involved in host specificity and recognition of target cell	Engel et al., 1983 Pilacinski et al., 1984	Encodes minor capsid antigen.	

product from the conserved sequences of BPV-1 , HPV-1 and HPV-6 was remarkably similar to that of polyoma and SV40 large T antigen. Similarly the E4 of HPV-8 showed homology with EBV-EBNA 2, necessary for the immortalisation of B lymphocytes by EBV. A similar function could explain the frequent malignant conversions associated with HPV-8 (Fuchs et al., 1986). Recently the E7 gene was shown to have a similar function to Adeno E1a in transactivating the Adeno E2 gene (Phelps et al., 1988). Since the E1a gene product can complex with retinoblastoma gene product to disrupt its tumour suppressor effects (Whyte et al., 1988), it could be that HPV E7 ORF has a similarly disruptive effect on normal cellular control functions.

This mechanism of gene regulation is conserved between PVs since BPV E2 can transactivate the expression of HPV genomes, with the E2 product binding directly to the conserved enhancer sequence, ACCG-CGGT (Phelps and Howley, 1987). Very recent extension of these findings has shown that different E2 proteins transactivate transcription by a common mechanism (Giri and Yanif, 1988). Not only did the E2 of HPV-18 or BPV-1 transactivate the LCR of CRPV, but the E2 of CRPV acted similarly on the LCR of BPV-1, HPV-1 and HPV-18.

2.32 Gene Products

The 31% non-transforming genomic fragment containing only two large ORFs is considered to encode the structural proteins found in productively infected cells. By indirect evidence from amimo acid analysis, the major capsid protein of BPV-1 particles was assigned to the L1 ORF (Meinke and Meinke, 1981) and this was confirmed by finding that sera raised against disrupted BPV-1 particles would react with synthetic L1 but not L2 (Pilacinski et al., 1984). Since BPV-1 virion antigens also react with antibodies to L2, it was considered that L2 also encoded a viral structural polypeptide (Pilacinski et al., 1984).

Extensive studies by Western blotting with antisera produced

against individually expressed ORFs of HPV-1a have shown that the major virion polypeptide of MW 57,000 (and equivalent to VP1 of Favre et al., 1975 and protein 2 of Gissmann et al., 1977) was the primary product of L1 (Doorbar and Gallimore, 1987). Very low levels of proteins of MW 50,000 and 43,000 (VP2 and 3 or proteins 3 and 4 of the earlier studies) were shown to be breakdown products only occasionally detected, with their frequent detection in early studies being due to the use of trypsin in virus purification. A polypeptide of MW 78,000 (and equivalent to the 76,000 polypeptide noted by Orth et al., (1977) or the 86,000 protein reported by Gissman et al., (1977)) was considered to be the product of L2, with a 65,000 moiety as its occasional breakdown product (Doorbar and Gallimore, 1987).

At the amino acid level, the L1 product is rich in cysteine (Chen et al., 1982). This protein is highly conserved between different PVs (Danos et al., 1982; Schwarz et al., 1983; Giri et al., 1985; Seedorf et al., 1985; Fuchs et al., 1986) and the resultant covalently linked subunits give a molecule which is resistant to SDS disruption. The L2 product, on the other hand, lacks cysteine and dissociates readily in SDS. As a result, while antisera to both L1 and L2 produce similar staining patterns in sections of warts, only the antiL1 serum will cross-react with other PVs. The use of SDS-disrupted BPV particles to produce a group-specific antiserum is therefore readily explained by the lack of dissociation of conserved L1 and the dissociation of non-conserved, type-specific L2.

It has long been known that the purification of virions from infected tissue may yield heavy full (buoyant density 1.36 g/ml) and light full (1.34 g/ml) as well as empty (1.29 g/ml) particles (Pass and Maizel, 1973) and it has recently been suggested by Mose-Larsen et al. (1987) that in BPV-1 at least, the heavy full particles contain a glycosylated form of L1. The increase in molecular mass is associated with charge modifications of L1 thought to be necessary to reduce the conformational stress between adjacent subunits

on the 5- and 6- fold axes of symmetry. Doorbar and Gallimore (1987) suggested an alternative reason for heavy full particles might be the presence of larger amounts of the heavier L2 in such particles.

Yet another explanation for heavier particles may come from the presence in highly purified HPV-1 virions of E4 proteins. Although described as an early gene, E4 products have many of the characteristics of late proteins and are found in abundance in HPV-1 induced lesions, representing as much as 20% of total wart protein content (Doorbar et al., 1986). These products are associated with cytoplasmic and nuclear inclusions (Croissant et al., 1985) and electrophoretic analysis shows doublets of 16 and 17,000 MW and of 10 and 11,000 MW (Doorbar et al., 1986). The 17,000 MW moiety is synthesised deep in wart tissue and as it decreases it is replaced by the 16,000 product. In the middle layers, antigenically related species of various sizes are found, probably as a result of post translational modification, and in the superficial layers, the 10 and 11,000 species predominate (Breitburd et al., 1987). The existence of E4 proteins as doublets, their persistence in the upper layers and their accumulation in granules is reminiscent of filaggrin, a component of keratohyalin granules (Breitburd et al., 1987) and indeed, E4 products do bind to filaggrin by electrostatic attraction (Doorbar et al., 1988).

Although the function of E4 proteins is unknown, their production in parabasal layers of productively infected cells suggests a role in virion maturation (Doorbar et al., 1986) while its great abundance in HPV-1 lesions and not in others may reflect an influence on the amount of virus produced (Breitburd et al., 1987). Recently Doorbar and his colleagues (1988, personal communication) have postulated that the binding of E4 and filaggrin prevents the formation of a cross-linked keratin matrix in superficial cells, possibly allowing virus to escape more readily from fragile cells.

The search for specific gene products of PVs has gained momentum as the transforming potential of both BPV and HPV has been

exploited. The ready availability of transformed cervical cell lines containing HPV such as HeLa (HPV-18), CaSki (HPV-16; Patillo, 1977; Yee et al., 1985) and SiHa (HPV-16; Friedl et al., 1970; Yee et al., 1985) has enabled specific mRNAs and their translated products to be identified]. Thus, the E2 of CRPV has been detected equally in both benign and malignant tumours (Wettstein et al., 1987) as a highly phosphorylated protein of MW 42000, while the E5 gene product of BPV-1 is a very hydrophobic protein of 44 amino acids associated with cell membranes (Schlegel et al., 1986). E5 is the smallest viral transforming protein yet characterised. The E6 protein of BPV-1 can be detected in both the nuclear and membrane fractions (Androphy et al., 1985). It has a MW of 15,500 and its regularly spaced Cys-x-x-Cys sequences reveal possible zinc ion binding.

Transcription of E1 was shown by Pater and Pater (1985) but the major transcripts in cervical carcinoma cell lines and in biopsies of cervical carcinomas are from the E6/E7 region (Smotkin and Wettstein, 1987; Shirasawa et al., 1987) and protein products of E6 and E7 have been found in both (Seedorf et al., 1987; Smotkin and Wettstein, 1987; Banks et al., 1987) although they are not always detected (Lehn et al., 1985; Pater and Pater, 1988). Indeed, a very recent report suggests that continued expression of E7 is essential for the maintenance of the transformed phenotype (Crook et al., 1989). Recent work suggests that the 20,000 E7 protein is a cytoplasmic phosphoprotein with similar zinc binding sequences as E6, abundantly produced but with a half-life of about 1 hour (Smotkin and Wettstein, 1987), while the 16,500 E6 polypeptide may be expressed only at very low levels (Banks et al., 1987).

Recognised gene products in PV systems are summarised in Table 3.

Table 3. Gene Products in PV Systems

ORF	Gene product in BPV transformed cells	Reference	Gene product in other PV systems	Reference
E1	'R' polypepetide - positive factor in genome replication 'M' polypeptide - phosphorylated modulator	Schiller, 1987		
E2	Transactivating enhancer element. Smaller repression protein	Phelps & Howley, 1987 Haugen et al., 1987	Highly phosphorylated protein found equally in benign and malignant CRPV 50,000 E2 protein found in 16 out of 24 condylomata acuminata	Wettstein et al., 1987 Li et al., 1988
E4			Proteins of 16-17,000 and 10-11,000 expressed abundantly in cytoplasm of terminally differentiated keratinocytes of HPV-1 lesions. 17,000 protein may have part of E1 spliced on	Doorbar et al., 1986 Breitburd et al., 1987 Doorbar et al., 1988
E5	Very hydrophobic small protein of 44 amino acids, associated with cell membranes, 34% leucine	Schlegel and Wade-Glass, 1987	Deer PV and European elk PV have E5 product similar to that of BPV, but many HPVs do not	Schiller 1987
E6	Highly basic polypeptide of 15,500 with regularly spaced Cys-X-X-Cys sequences, creating nucleic acid binding looks stabilised by zinc linked through cysteines. Found in both cytoplasm and nucleus	Androphy et al.,1985 Giri and Danos, 1986	In HPV-16 lesions, protein of MW 16,500 expressed at low levels or not at all	Banks et al., 1987 Lehn et al., 1985 Pater and Pater, 1988
E7	Nucleic acid binding protein with similar structure to E6		Abundantly expressed 20,000 protein which undergoes serine phosphorylation, found in cytoplasm of HPV-16 and HPV-18 cell lines	Smotkin & Wettstein, 1987
L1	Protein of MW 55,000 highly acidic, rich in cysteine, forming major capsid protein	Meinke & Meinke, 1981 Doorbar & Gallimore, 1987	Conserved between different PVs	
L2	Protein of MW 78,000, lacking cysteine forming type specific capsid protein	Doorbar & Gallimore, 1987	Not conserved between PVs	

3. Pathogenesis of Papillomaviruses

3.1 General Biological Properties of PVs

Infection of cutaneous or mucosal epithelium with papillomaviruses induces squamous tumours in a wide variety of animals. Such tumours usually show limited growth and often regress spontaneously (Massing and Epstein, 1963). Productive infection occurs only in fully differentiated squamous cells and electron microscopical analysis of cutaneous warts reveals virions in increasing numbers from the stratum granulosum through the stratum spinosum and eventually to crystalline arrays within the nuclei of degenerating keratinocytes (see Cubie, 1972a).

It is generally believed that the virus infects basal cells of the epidermis following minor abrasions of the skin as has been shown for CRPV (Nasseri and Wettstein, 1984a) and establishment of infection may depend on the activation of cell division during healing. The expression of early viral genes to promote proliferation and delay activation of superficial keratinocytes leads to the characteristic hyperplasia of warts. The control of late gene expression, however, is tightly linked to the state of differentiation of the squamous cells. Thus, while in situ DNA or RNA hybridisation may reveal the presence of papilloma genomes and transcripts in the parabasal layers, structural proteins and particles are only found in the permissive terminally differentiated cells of the upper layers.

3.2 Recent Attempts to Produce Models of PV Infection in the Laboratory

3.21 Cell Culture Studies

Despite the lack of success of early cell culture experiments with HPV (see Cubie 1972a; Cubie, 1974), attempts continued to be made to detect HPV activity in cultured cells. Butel (1972) obtained

transient stimulation of cellular DNA synthesis 24 hours after infecting foetal rabbit kidney cells with plantar wart virus, and in a similar experiment, Lancaster and Meinke (1975) obtained transient DNA synthesis in human lung fibroblasts after infection with HPV. Their cultures continued to contain a stable amount of HPV-DNA, equivalent to about 0.2 viral genomes/cell, throughout 40 cellular doublings. The cells were not transformed however and had the same life span as uninfected controls. In 1975 Eisinger and colleagues successfully infected a human epithelioid cell line with HPV from hand warts under condition of pH stress. The cells appeared to produce virus particles over many passages. This is the only record of a productive HPV infection in vitro, but the cell line died out and the experiment could not be repeated. (Eisinger, personal communication).

Once it was realised that PV viral replication and virion assembly was only found in cells which were keratinising, interest switched to the culture of keratinocytes for HPV propagation. Direct explant culture of the keratinocytes from epidermal warts (Niimura et al., 1975) laryngeal warts (Steinberg et al., 1982) and anogenital warts (Rose et al., 1987) has been described, but no evidence of vegetative growth was obtained. La Porta and Taichman (1982) successfully infected human foreskin keratinocytes grown on irradiated NIH 3T3 feeder layers with HPV from plantar warts to produce a cell-line in which HPV-DNA persisted at 50-200 copies/cell and replicated as a stable episome for up to 8 passages, but with no evidence of virus replication or cell transformation. Viral DNA replication occurred only in the proliferating cells and not in cells undergoing differentiation. Perhaps the use of epidermal growth factor, and cholera toxin to encourage proliferation prevented progression to vegetative production in the differentiated cells, or perhaps because HPV-1 does not normally infect genital epithelium, replication was blocked (Taichman et al., 1984).

While no model for vegetative replication of HPV has yet evolved, the transformation of rodent cells with HPV of various types is

now well documented. Successful tranformation with focus formation of C127 cells by HPV-1 and HPV-5 DNA was reported by Watts et al., (1984). However, the HPV-1 clones grew poorly in soft agar and produced no tumours in nude mice, whereas HPV-5 clones showed both anchorage independence and tumorigenicity. DNA remained as multiple persistent episomal copies. These results with HPV-1 DNA have not been reproduced by others, although some success with HPV-8 cloned fragments from the E6 region was recently reported when the transformation was driven by a retroviral LTR. (Iftner et al., 1988). Cloned HPV-1, 2, 3, 4 and 9 were successfully transfected with the HSV thymidine kinase (tk^+) gene into mouse L cells lacking tk. Integration of the HPV-DNA into the host genome occurred in all cases (Brackmann et al., 1983). Similarly, co-transfection of HPV-1 DNA with origin-minus SV40 into rat fibroblasts or human keratinocytes led to integration of the HPV-1 (Burnett and Gallimore, 1983, 1985). It should be noted, however, that co-transfection with dominant markers may make the recognition of transformed cells easier, but it may also per se encourage integration of those HPV types considered to be less oncogenic.

HPV-16 DNA or specific fragments containing the E6/E7 ORFs have been shown to transform mouse 3T3 cells (Yasumoto et al., 1986), rat 371 cells (Kanda et al., 1988), and primary rat cells in conjunction with activated ras gene and a retroviral LTR (Matlashewski et al., 1987) or activated ras in the presence of dexamethasone (Pater et al., 1988). HPV 16/18 DNA can also transform human foreskin fibroblasts and human epithelial cells (Pirisi et al., 1987; Kaur and McDougall, 1988; Matlashewski et al., 1988) and the transforming activity has been localised to the E6/E7 region of the HPV-16 genome (Storey et al., 1988; Woodworth et al., 1989; Bedell et al., 1989). Di Paolo et al., (1987) noted that cells transformed by HPV-16 underwent drastic chromosomal alterations including deletion of chromosome 12 in the region of the Ki-ras-2 gene. Progressive integration of HPV sequences during

transformation was noted. Although transformed, the transfected human cells were not tumorigenic in nude mice, while the mouse cells were; this suggests that HPV-16 has oncogenic potential but that a unique interaction between viral DNA and the host cell is necessary to establish tumorigenicity with specific integration being required for progression to the fully malignant state. Recently Popescu et al. (1987) reported a cell line in which multiple copies of HPV-18 were integrated at a single heritable fragile site on chromosone 12 and noted that in Hela cells, HPV-18 is integrated at four sites, three of which are at or in close proximity to fragile sites.

Transfection of human cells with HPV provides a useful in vitro model to study the molecular biology and oncogenic potential of specific HPV types in a human background. Refinements to the techniques will bring a greater awareness of specific host-virus interactions.

3.22 Animal Models

Attempts to produce an animal model of vegetative replication of HPV have until recently been unsuccessful. In 1971, Kreider et al. failed to induce papillomas in human skin transferred to the immunologically privileged hamster cheek pouch after infection with wart extracts and in 1973, Pass et al. similarly failed to produce papillomas when infected human skin was grafted onto mice treated with anti-lymphocytic serum. Although Cubie (1976) induced small nodules containing a few intranuclear virus-like particles on human skin exposed to a purified extract of simple plantar warts and grafted onto athymic "nude" mice, no true papillomas developed over a 10-week observation period. Kreider and his colleagues (1979), on the other hand, were able to obtain neoplastic transformation of normal rabbit skin grafted to nude mice with CRPV. Kreider continued to exploit the nude mouse system and eventually in 1985 reported the successful growth of condylomata acuminata containing HPV-11 when fragments of normal cervical tissue were suspended in an extract of genital warts and then

implanted under the renal capsule of the nude mouse (Kreider et al., 1985). Extension of this work suggested that the most vigorous proliferative response to HPV-11 was engendered by the use of split-thickness human foreskin rather than cervical epithelium (Kreider et al., 1986). Virus from the experimental condylomata was successfully used to infect fresh foreskins and it was possible to produce sufficient HPV-11 from experimental grafts to purify the virions on cesium chloride gradients (Kreider et al., 1987). Because the condylomata develop as cysts with all the keratin layers contained in the core of the cyst rather than being desquamated as in natural lesions, a rich source of virions is retained. Approximately 50% of experimental infections resulted in cyst formation. This unique system proves that transformation of human tissues by papillomaviruses under controlled experimental conditions is possible.

Other small rodents might also provide suitable animal models for the study of PV. In the 1970s, a papillomavirus was found in epithelial proliferations, particularly keratoncanthomas, of Mastomys natalensis (MnPV; Muller and Gissmann, 1978). Some colonies of Mastomys harbour endogenous MnPV and this is associated with a high rate of stomach carcinoma. It is thought that food additives might act as co-factors in tumour induction (Amtmann et al., 1984). A closely related virus has recently been described in naturally occurring papillomas and viral DNA was found in several different types of skin lesions in the European harvest mouse, Micromys minutus (Sundberg et al., 1987). Because viral DNA was also found in 21/34 histologically normal skin biopsies it was suggested that this virus would remain endogenous and be transmitted vertically in much the same way as MnPV (Sundberg et al., 1987). Endogenous PV genomes are also thought to be responsible for the papillomas readily induced in hairless mice with low doses of UV light. Progression from papilloma to carcinoma-in-situ and to squamous cell carcinoma has been observed, and sequences related to MnPV were detected in 36% of tumours (Gallagher et al., 1984; Tilbrook et al.,1989).

3.3 Morphological and Histological Characteristics of HPV Lesions

3.31 Cutaneous Sites

It is well recognised now that certain types of HPV are associated with particular morphological characteristics, but the association is not absolute (Jablonska et al., 1985b). Broadly speaking, skin warts can be divided into three groups :-

(i) Common warts which are usually exophytic, multiple, irregular nodules often on the hands, or superificial spreading mosaic-type warts on the feet. (Figure 4a and b). They are usually caused by HPV-2 (Laurent et al., 1982) although the smaller endophytic lesions may also be caused by HPV-4 (Jablonska et al., 1985) and the hyperproliferative lesions common in butchers by HPV-7 (Orth et al., 1983). HPV-7 has recently been reported to be common in fishmongers too (Rudlinger et al., 1989) with the chronic maceration of the skin of the hands of both groups due to moisture and cold, possibly contributing to infection. A similar situation appears to exist in poultry workers (Guillet, 1987) although the virus type found in these workers has yet to be identified.

Histologically, common warts show prominent papillomatosis acanthosis, hypergranulosis and hyperkeratosis. Within granular cells of HPV-2 lesions there is often a marked clearing of the cytoplasm, with numerous keratohyalin granules and HPV-4 have more pronounced crescentic nuclei (Jablonska et al., 1985).

(ii) Myrmecia (Figure 4c) or deep, painful endophytic warts surrounded by a horny ring, commonly but not always found on the plantar surface of the foot and always induced by HPV-1. They have a peak incidence in children aged 12-15 (Pfister and zur Hausen, 1978).

In the electron microscope, HPV-1 lesions contain densely

Fig 4. Typical cutaneous lesions due to HPV

(a) Common hand wart (b) Mosaic plantar wart
(c) Myrmecia or simple plantar wart (d) Plane wart
(e) Condylomata acuminata
(originals kindly provided by Dr. M.H. Bunney).

packed virus particles often in crystalline array and it is from such lesions that much of the early virus preparations were made (Cubie, 1972a). Histologically, they have an almost totally disorganised granular layer due to their endophytic growth. Clear cytoplasm with eosinophilic inclusions are prominent in the stratum granulosum, the eosinophilic inclusions being associated with the two low molecular weight polypeptides (of 16,000 and 17,000) unique to HPV-1 lesions (Breitburd et al., 1987). Parakeratotic cells in the upper layers contain nuclear basophilic inclusions, and it is these that contain the virions.

(iii) Plane warts which are slightly raised, flat topped small lesions often on the face, occurring as multiple crops. (Figure 4d). They are usually caused by HPV-3 or HPV-3 related types such as HPV-10 (Orth and Favre, 1985). Papillomatosis in these lesions is slight, but perinuclear vacuolisation of cells of the upper layers is again evident. In the EM, this is shown to be due to the accumulation of keratin filaments at the cell periphery, giving a net-like structure in which pyknotic nuclei are centrally suspended. No free keratohyalin granules are observed (Croissant et al., 1985).

Plane warts exhibit a characteristic phenomenon of spontaneous regression of the whole crop, with all lesions becoming involuted within 2-6 weeks. (Tagami et al., 1977), in total contrast to the absence of lymphocyte cell infiltration in common and plantar warts (vide infra).

A variety of cutaneous warts develops in people suffering from epidermodysplasia verruciformis (EV) which is thought to be linked to a rare recessive abnormal allele of an autosomal gene (Orth, 1986a). Selective and partial defects of cell mediated immunity (Jablonska, 1986) in these patients are associated with the development of disseminated skin warts which persist throughout life. Many of the lesions contain

HPV-like particles (Ruiter and van Mullem, 1966), resemble flat warts and are localised on the extremities and face. They may be induced by HPV-3 or HPV-10 as in the general population or by a whole series of HPV types only found in EV patients (Kremsdorf et al., 1983; 1984). Other lesions resemble the reddish plaques of pityriasis versicolor rather than warts (Jablonska & Orth, 1985a). These lesions are induced by HPV 5, 8 and 9 and exhibit a common histological picture. The beautiful studies of Croissant (Croissant et al., 1985) show that viral DNA-containing cells found in groups or columns have a uniformly pale cytoplasm, while more superficial cells look clear due to the loss of tonofilaments. Prominent tiny drops of keratinohyalinlike material can be observed and virions are packed round the margin of the nuclei of the upper granular layers close to condensed chromatin.

About one-third of EV patients develop multiple skin carcinomas, approximately 25 years after the development of benign lesions, with malignant conversion occurring at light exposed sites (Jablonska et al., 1972). The carcinomas harbour no HPV-like particles (Ruiter and van Mullem, 1970) but only HPV-5 and some HPV-5 related DNA sequences (e.g., HPV-8, 14, 17, 20) (Ostrow et al., 1982; Pfister et al., 1983a; Kremsdorf et al., 1984; Lutzner et al., 1984; Pfister, 1987b), whereas benign lesions in the same patients can contain many different types, suggesting a role for HPV-5 in the development of neoplasia in EV patients (vide infra). Conversely, HPV-3 lesions do not become malignant (Orth et al., 1979).

Skin cancers in EV patients are discussed more fully in Section 3.54 and the immune defects in such patients in Section 4.4.

3.32 Muco-cutaneous sites

Genital warts or condylomata acuminata occur in men on the penis and anus and in women on the vagina, vulva and perineum as

small verrucous papules or as raised multiple lesions resembling common warts, or even as hyperplastic pedunculated cauliflower-like masses (Oriel, 1971b; Figure 4(a)) Human papilloma virions can occasionally be found in small numbers in such lesions (Dunn and Ogilvie, 1968) and more than 90% of German cases have been shown to be caused by HPV-6 and the related HPV-11 (Gissmann et al., 1982a). Malignant conversion of condylomata acuminata is well documented with Boxer and Skinner noting 65 cases in the literature, 8 of which had metastatic involvement (Boxer and Skinner, 1977).

Histologically, condylomata show epithelial thickening due to extreme acanthosis and papillomatosis but without the hyperkeratosis of external cutaneous warts. Cells of the stratum spinosum become several times larger than normal with distinct perinuclear vacuolisation (Grussendorf-Conen, 1985). A chronic inflammatory infiltrate is usually observed.

Warts of the throat or laryngeal papillomas are the most common benign neoplasm of the larynx in children (Steinberg and Abramson, 1985) although they also occur with similar aetiology in adults. Laryngeal papillomas in children can easily block the small airway, making surgical intervention essential and life-saving, but recurrences are frequent and children can require surgery every four weeks or so (Steinberg et al., 1983) with spread from the surgical trauma a well recognised complication (Duff, 1971). The papillomas have a thickened spinous layer with increased nucleoli, and there is a delay in maturation of the upper layers with a lack of keratohyalin and a loss of superficial flattening (Steinberg and Abramson, 1985). It had long been suggested that laryngeal papillomas were another manifestation of wart virus infection, but proof was lacking until recently.

HPV common antigen was found by Lack and his colleagues (1980) in a few superficial cells in about half of the tissues examined and by careful reprocessing of tissue showing antigen positivity,

papilloma-like particles with nuclear localisation were found. In addition, HPV DNA subtypes related to HPV-6 were shown to be present by Southern blot analysis (Mounts et al., 1982). About 50% of laryngeal papillomas examined by Gissmann's group in Germany contained the closely related HPV-11 (Gissmann et al., 1982, 1983) and more recently all ten cases examined in Leeds were positive for HPV-6 or HPV-11 (Terry et al., 1987). Not only have HPV DNA sequences been found in the laryngeal papillomas themselves but also in normal tissue adjacent to papillomas and in normal tissue from previous papilloma patients in remission, suggesting that the HPV DNA can persist with no clinical sign of disease (Steinberg et al., 1983).

There is some epidemiological evidence to link laryngeal papillomas with genital condylomata, with the suggestion that genital wart virus is acquired from the mother during birth, leading to the development of laryngeal papillomas after a variable latent period. In the series presented by Quick et al., (1978), 60% of children with laryngeal papillomas had a maternal history of condylomata acuminata. Conversely, only 2/23 children in the study from Steinberg and Abramson (1985) had a positive maternal history and, furthermore, two children who developed laryngeal papillomas in the first few weeks of life, were born by Caesarean section.

Maternal transmission to the infant has likewise been assumed in early cases of condylomata acuminata in childhood (Patel and Groff, 1972), but Roman and Fife (1986) could not correlate the finding of 3/70 HPV positive lesions in newborn foreskin with maternal abnormalities. The possibility of latent infection will be discussed below.

Other lesions of the oral cavity, particularly focal epithelial hyperplasia of Heck (FEH) found in Eskimos and American Indians (Praetorius-Clausen, 1972) have also been shown to be associated with HPV. HPV-13 has been found in FEH (Pfister et al., 1983) and HPV-2 in common warts of the oral mucosa (Lutzner, et al., 1982).

Papillomatous lesions frequently carry HPV-11 related types (Naghashfar et al., 1985; Loning et al., 1985) while HPV is variably found in other oral lesions and tumours (vide infra, Section 3.55).

The interesting co-existence of EBV and HPV in cells from lesions of oral hairy leukoplakia in AIDS patients was described (Greenspan et al., 1984) but has not been confirmed by others.

Mucosal epithelium of the oral cavity and larynx, like the epithelial surfaces round the genital area, differ from external skin in the degree of keratinisation which is present. Yet HPV replicates only in terminally differentiated cells and it is understandable that papilloma virus antigens, and even more so, typical papilloma particles, are found only rarely and scantily in these lesions. Nevertheless, the search for HPV DNA sequences has revealed the association between papilloma viruses and laryngeal and genital papillomas, thus confirming a long-standing clinical belief.

3.33 Dysplastic Lesions of the Genital Tract

Morphological changes similar to those seen in HPV infections were first observed in smears and sections of the uterine cervix by Meisels and Fortin (1976) and Purola and Savia (1977), often in association with cervical intraepithelial neoplasia (CIN). Tiny flat warts, usually in the tranformation zone where cell division is more rapid were revealed following application of acetic acid and magnification with a colposcope (Reid et al., 1980). These lesions have been variously named non-condylomatous wart virus infection (NCWVI - Reid et al., 1980), flat condylomata or condylomata plana (Meisels et al., 1977) and they are almost indistinguishable colposcopically from mild cervical dysplasia or CIN. Histological examination of punch biopsies from such lesions show a slightly thickened epithelium and the presence in the superficial layers of "koilocytes" i.e., ballooned cells with distorted, pyknotic nuclei and a large perinuclear space (Koss and Durfee, 1956). A subset of CIN lesions where the changes are more

severe with koilocytes covering the whole thickness with disordered stratification and bizarre hyperchromatic nuclei were called atypical condylomata (Meisels et al., 1981) or type 2 CIN lesions (Pilotti et al., 1981).

It was the similarity of koilocytes to the vacuolated enlarged cells of the granular layers of many types of wart which prompted a search for HPV in cervical lesions and the presence of koilocytes is now considered pathognomonic of HPV infection. Such a presence provided histological evidence of HPV infection in more than 90% of cases of CIN in Australian women (Reid et al., 1982). Confirmation of a viral aetiology again came from the finding of papillomavirus-like particles in the nuclei of koilocytes (Laverty, 1979; Hills and Laverty, 1979; Pilotti et al., 1981) and indeed Laverty found particles easier to detect in NCWVI than in condylomatous lesions. Further confirmation came from the detection of papilloma antigen in similar superficial cells using antiserum against the group antigen (Woodruff et al., 1980; Morin et al., 1981) and the demonstration of HPV DNA sequences most frequently of types HPV 6, 11 and 16 (Gissmann et al., 1983; McCance et al., 1983) in the deeper layers. It was suggested that HPV was capable of establishing a permissive, productive infection only in differentiated superficial cells and a non-permissive infection in the deep layers which might then undergo transformation to dysplasia. (Fletcher and Norval, 1983).

Because of the difficulties in distinguishing NCWVI from CIN, searches have been carried out in many countries for the presence of HPV DNA sequences in CIN of all grades of severity, including carcinoma in situ (now classified as CIN III) and invasive carcinoma. It soon became obvious that HPV-16 (Durst et al., 1983) and to a lesser extent, HPV-18 (Boshart et al., 1984) were preferentially associated with severe dysplasias and cancer. A selection of prevalence surveys in various populations where it has been possible to compare the CIN gradings from biopsies, smears or cervical swabs and match them with the HPV type found, are shown in Table 4. Although there is

Table 4 Prevalence of different HPV-types associated with CIN and cervical carcinoma

HPV type	Normal tissue 6/11	16/18	No. of patients	CIN I and II 6/11	16/18	No. of patients	CIN III 6/11	16/18	No. of patients	Invasive carcinoma 6/11	16/18	No. of patients	Country of origin of cases	Reference
	.	.	.	.	.	.	.	.	.	.	61%	18	Germany	Durst et al., 1983
	.	.	.	.	.	.	.	.	.	.	35%	23	Kenya & Brazil	"
	.	.	.	67%	.	13	67%	.	6	.	.	.	UK	McCance et al., 1983
	11%	0	36	33%	33%	25	18%	68%	22	.	.	.	Germany	Wagner et al., 1984
	0	18%	17	25%	62%	50	*	71%	28	0	90%	13	UK	McCance et al., 1985a
	.	.	.	25%	40%	45	8%	57%	22	0	100%	4	Germany	Schneider et al., 1985
	.	35%	26	.	39%	100	.	.	.	.	66%	47	UK	Meanwell et al., 1987
	5%	0	21	13%	28%	39	0	100%	2	0	59%	39	USA	Lorincz et al., 1987+
	0	0	2	7%	28%	29	0	50%	8	0	75%	8	Brazil	"
	.	.	.	.	.	.	.	.	.	0	71%	17	Peru	"
	2%	44%x	43	0	75%x	36	0	80%x	5	.	.	.	USA	Schneider et al., 1987
	29%	34%x	35	24%	59%x	58	8%	71%x	45	0	64%x	25	Germany	" " "

* HPV - 6/11 only found in this group in association with HPV-18

\+ An additional type, HPV-31 was included in this American study, since this type had been shown to occur in about 20% of CIN I and II and 6% of carcinomas in the USA (Lorincz et al 1986).

x These figures include lesions in which HPV 6/11 was found in addition to HPV 16/18

considerable variation in the percentages obtained by each group of workers, there is an obvious increase in the percentage of HPV 16/18 positive cases as the severity of the lesion increases. These findings all add weight to the speculation that infection with HPV-16/18 might increase the risk of development of a malignant tumour (vide infra). However, they do not represent full epidemiological investigations : some do not include controls or do not describe the controls adequately to prevent selection biases (Munoz et al., 1988); some use biopsies and others cytological specimens which cannot readily be compared because of sampling variation, yet no study has been made which compares the results from biopsies and cytological specimens from the same patients. Furthermore, different methods of DNA detection with widely differing sensitivities have been used and analysis of other risk factors in comparisons between groups have not been made. Finally, the increased finding of HPV sequences in apparently normal cervices in recent studies, after laser ablation (Wickenden et al., 1985), in the normal epithelium at a distance from observable lesions (Ferenczy et al., 1985) and in control populations (Cox et al., 1986; Macnab et al., 1986; Meanwell et al., 1987; Reeves et al., 1987; Schneider et al., 1987) suggests that latent HPV infection requires other risk factors for progression.

A histological pointer of increased risk was noted by Crum et al. (1984) in a study of "flat condyloma" in which lesions were divided according to the presence or absence of abnormal mitotic figures (AMF). 70% of lesions with AMF were found to harbour HPV-16 while 77% without AMF did not. Thus the suggestion was made that lesions containing both AMF and HPV-16 were the most likely to progress. The association between these factors was recently confirmed (Ionesco et al., 1987). In this study, 7 out of 13 HPV-16 positive flat condyloma and 20 out of 30 HPV-16 positive CIN lesions all also showed AMF and/or basal and parabasal cell nuclear atypia. The presence of AMF suggests aneuploidy, a recognised characteristic of malignancy.

Jakobsen (1983) and Reid et al. (1984) showed that increasing aneuploidy occurred with increasing severity of CIN but using flow cytometry, Hughes et al. (1987) found similar proportions of aneuploid cells not only in all grades of CIN, but also in lesions which had histological evidence of papillomavirus infection in the absence of CIN. HPV typing of these lesions, however, was not done, and it might be valuable to consider AMF, aneuploidy and HPV type in the same lesions.

Intraepithelial neoplasia of the external genitalia, or Bowenoid papulosis, showing as small flat often inconspicuous papules on the penis or as multiple confluent, elevated and often pigmented lesions of the vulva and perineum in women (Obalek et al., 1985) are similarly associated with HPV-16. Bowen's disease itself is regarded as carcinoma in situ and is found in older men. HPV-16 was found in 8/10 German cases (Ikenberg et al., 1983) and 12/14 Polish cases (Obalek et al., 1985). Papillomavirus-like particles were observed in individual cases of Bowenoid papulosis (Obalek et al., 1985) and a few cases have been HPV antigen positive (Braun et al., 1983; Gross et al., 1985a). Histologically, hyperplasia with numerous AMF and large dyskeratotic cells is seen, and, like the HPV-16 containing CIN lesions, koilocytes are absent. Bowen's disease and cervical neoplasia are often found to co-exist (Hilliard et al., 1979).

Given the various terminologies currently in use, Gross et al. (1985b) called for a new nomenclature for papillomavirus infection in the anogenital region, dividing the lesions into three distinct clinical groups: condylomata acuminata, flat cervical warts and pigmented papules. They stressed the lack of correlation between koilocytic type, clinical type or virus type, with the exception that HPV-16 was frequently found in non-koilocytic lesions with severe epithelial atypia including AMF and an absence of structural antigen. The limitations and uses of different methods of classification are shown in Table 5, and cervical flat warts showing moderate dysplasia are depicted in Fig. 5.

Table 5. Limitations and uses of cervical HPV infection and CIN classification methods (from Grubb, 1986)

Method of classificatiion	Limitation of method	Epidemiological uses	Epidemiological limitations
Papanicolaou smear	Difficult differentiation between HPV and CIN I	Initial screen for HPV and and CIN	Low sensitivity in the general population
Immunoperoxidase staining of capsid antigen	Detects HPV antigen in 50% of cervical warts	Highly specific confirmatory test of HPV infection	Low sensitivity; Time-consuming
Electron microscopy	Detects HPV virions in 25-50% of cervical warts	Complementary confirmatory test to immunoperoxidase test	Low sensitivity; Time-consuming
Cell ploidy determination	Difficult differentiation between aneuploidy & extreme polyploidy	Identified atypical HPV lesions and CIN more likely to progress	Requires expensive equipment; Time-consuming
Abnormal mitotic figures (AMFs)	Not specific to CIN; Difficult to detect different AMF types	Identifies atypical HPV lesions and CIN more likely to progress	Requires cytological expertise; Time-consuming
HPV DNA typing	Not all HPVs can be identified with known HPV DNA types at present	Sensitive and highly specific for classification of HPV	Requires laboratory expertise and known HPV DNA types (probes)
Colposcopy	Difficult differentiation of HPV infection from CIN I; cannot see endocervical canal	Monitor lesion without affecting progression	Requires colposcopic expertise; Classification criteria not standardized
Serology	Not specific to genital HPV infections	Detects previous exposure to HPV	No current serologic test sufficiently specific

3.4 The Male Factor in HPV Transmission

Extensive epidemiological studies over the last 150 years since Rigoni-Stern (1842) noted the lack of development of cervical carcinoma in nuns have clearly shown that age at first intercourse and multiple sexual partners are risk factors associated with the development of cancer of the cervix (Rotkin, 1973; Singer et al., 1976) and of CIN (Harris et al., 1980). As a result, Singer and his colleagues suggested that a closer look should be taken at the male partner to identify "high risk males" (Singer et al., 1976). They proposed at the time that different levels of histones in the sperm head or of polyamines in seminal plasma might exert deleterious effects on the stem cells of the transformation zone of the cervix. Several infectious agents have been incriminated in the transmission of the disease, but without conclusive evidence (Alexander, 1973; In Vonka et al., 1984). particular, herpes simplex virus (HSV) type 2 seemed a likely agent, but the evidence was mainly serological (Rawls et al., 1973) and inconclusive, since varying percentages of patients in different areas developed cervical cancer in the absence of HSV antibodies.

Zur Hausen (1976, 1977) suggested that the localisation of genital warts, their venereal transmission and malignant transition provided overwhelming evidence to support the involvement of HPV in genital dysplasias and the concept of the "high risk male" as the vector of HPV infection with the woman as the host in whom cancer developed gathered momentum. This was confirmed by Campion et al. (1985) who found that one third of women who had been the sole sexual consort for at least one year of men with condylomata acuminata not only had similar genital lesions but also have cervical abnormalities. Similar HPV types were found in each pair of sexual partners. Although HPV types 6 and 11 are commonly found in penile condylomata acuminata and can readily be transmitted from these productively infected lesions, six of the nine men who were consorts of women with CIN also have HPV-16 in their lesions. Perhaps these men, in addition to their

obvious genital warts, had inconspicuous lesions of Bowenoid papulosis which were sampled at the same time. Certainly, Gross and his colleagues (1985c) suggest that it is these inconspicuous lesions in young males which act as the natural reservoir for HPV-16 and in which the virus can replicate, preferentially destroying infected cells and thus preventing progression to neoplasia, and at the same time providing a source of virus to infect the sexual partner (Gissmann & Schwarz, 1986). Support for this hypothesis came from the finding of HPV-16 DNA in a penile Bowenoid papule and a concurrent carcinoma in situ (CIS) in the sexual partner. (Hauser et al., 1985).

3.5 Malignant Transformation in Papillomaviruses

Several animal PVs are capable of inducing carcinomas, but in each system studied some component in addition to the PV itself appears to be required. In the earliest studied system, CRPV-induced papillomas rapidly progressed to carcinomas in the presence of chemical carcinogens such as tar (Rous and Friedewald, 1944). Bladder tumours in cattle are associated with BPV-2 and alimentary tract carcinomas with BPV-4, but in both of these systems, the ingestion of bracken fern contributes to the progression to malignancy (Lancaster & Olson, 1982). Carcinomas also develop round the eyes of cattle (Ford et al., 1982) and the faces and ears of sheep (Vanselow et al., 1982) in tropical and sub-tropical Australia where precursor lesions containing PV progress in association with chronic exposure to sunlight. In man too, the carcinogenic and immunosuppressive effects of uv-light cause progression of HPV-5 induced papillomas in patients with EV. Children whose laryngeal papillomas were treated by X-ray often developed laryngeal carcinomas in later life, and genital lesions induced by different HPVs progress to frank carcinomas in the presence of various mutagenic stimuli. All of these additional components share mutagenic and in some cases immunosuppressive properties which may act in conjunction

with an underlying PV infection to cause tumorigenic progression. In this section, the bovine papilloma system is described in detail as a model for HPV-induced genital tumour development and the CRPV papilloma-carcinoma complex as a model for HPV-induced cutaneous carcinogensis in EV patients.

3.51 The bovine papilloma model

Six distinct BPV types have been isolated and characterised and these can be divided into two sub-groups. Subgroup A (BPV-1, 2 and 5) induce cutaneous fibropapillomas while sub-group B (BPV 3, 4 and 6) infect only squamous epithelial cells (Jarrett et al., 1984). The different types are readily distinguished by the their restriction endonuclease cleavage patterns and their degree of DNA sequence homology. BPV-1 and BPV-2 have a wide tissue range in cattle and also show the broadest experimental host range exhibited by any PV (Lancaster and Olson, 1982). For example, fibrosarcomas can be induced in horses and in hamsters. These can metastasise to internal organs, expecially the lungs, and BPV can be found in large quantities (100-700 copies per cell) within them (Lancaster et al., 1976; Lancaster et al., 1977).

The tumours produced in vivo by BPV-1 and BPV-2 do not have obvious human counterparts, but they are of interest because the infection is non-permissive in the dermis and only at much later stages of infection do particles accumulate in the upper epidermal layers with lymphocyte infiltration finally leading to regression (Jarrett, 1985). BPV-1 has shown no potential for malignant progression in vivo despite transformation in vitro, but BPV-2 on the other hand can produce tumours of the urinary bladder when inoculated experimentally. An infective agent resembling bovine wart virus was noted in 6/16 suspensions prepared from spontaneous bladder tumours (Olson et al., 1965) but the major aetiological factor in the development of bladder tumours was the ingestion of bracken fern (Olson et al., 1969). Even

at this time, the carcinogenic and immunosuppressive effects of bracken were known and Olson suggested a co-operative action between bracken fern and virus in the development of neoplasia.

The viruses of the true epithelial papillomas in cattle, the sub-group B viruses, are different not only from sub-group A viruses but also from HPVs and CRPV. Their genomes are considerably smaller (MW 4.4 x 10^6 rather than 5 x 10^6) and have a sequence length of 7200 bp, with neither BPV-4 nor BPV-6 possessing the gene for the interspecies PV antigen (Campo and Coggins, 1982). BPV-4 is the best studied member of the group as a result of its association with upper alimentary tract papillomas and the development of alimentary carcinomas again following the ingestion of bracken fern.

Examination of large numbers of cattle, mainly from farms in Argyll infested with bracken (Jarrett et al., 1978) showed that 96% of those with carcinomas had multiple papillomas at adjacent sites, containing BPV-4 (Campo et al., 1980). Older papillomas contained less virus and were less successful in inducing papilloma formation in calves and indeed, experimental papillomas more than 9 months old were both virus and viral DNA free (Jarrett, 1987). It appeared that although required for the induction of papillomas, the presence of BPV-4 DNA was not necessary for progression to, nor maintenance of, the transformed state. (Campo et al., 1985).

Intradermal inoculation of BPV-4 into hamsters led to the growth of malignant sarcomas (Moar et al., 1986), and molecularly cloned BPV-4 DNA transformed NIH 3T3 cells and, less efficiently, C127 mouse fibroblasts (Campo and Spandidos, 1983). In both these systems, the BPV-4 DNA persisted as multiple copies of non-integrated monomers. In most cell lines established with linear DNA, the genome was no longer present and in the few which did contain viral DNA, this was in an integrated, rearranged state (Smith and Campo, 1988). Similarly, explanted cell lines originating from alimentary papillomas or carcinomas produced fully transformed epithelial cells, but neither they

nor the nude mouse tumours derived from them contained any viral DNA (Campo and Jarrett, 1987). It seems likely that BPV-4 acts by a "hit and run" mechanism. It executes an early event which results in cellular proliferation, thus increasing the target size for subsequent neoplastic events, perhaps with amplification of epidermal growth factor receptors (Smith et al., 1987) or with the activation of cellular oncogenes (Amtmann et al., 1987). In addition, the trauma caused by eating hard sharp bracken further increases the local epithelial mitotic rate and the chemicals within the fern could act as promoters long after the virus has vanished (Campo and Jarrett, 1987). If this model is correct, there is only an indirect involvement of BPV-4 in the process of alimentary canal carcinogenesis.

The interaction between BPV-4, bracken and immunosuppression is being investigated by Campo and Jarrett in a complex experiment whereby calves born of papillomatosis-free mothers were obtained at birth and reared in isolation (Campo and Jarrett, 1986). Calves inoculated with BPV-4 alone developed alimentary papillomas which eventually regressed. Animals given a regular bracken diet in addition to BPV-4 developed multiple papillomas which have persisted for years and at least one has progressed to malignancy, with no detectable BPV-4 DNA in the tumour. Several animals which received bracken feed only developed cutaneous warts caused by BPV-1 and BPV-2 at the sites of repeated venepuncture. While it is just possible that BPV-1 or BPV-2 carried on the footpads of flies would allow transmission of virus from the wild, by analogy to the suggested transmission of CRPV by insects (Dalmat, 1958), it seems more likely that these viruses were persisting in a latent form, becoming activated by skin damage and immunosuppression (Campo and Jarrett, 1987).

Circulating lymphocytes have been suggested as a possible site of latency and indeed BPV-2 DNA was found in lymphocytes of four animals given bracken, BPV-1 DNA in two animals given virus and both BPV-1 and BPV-2 DNA in some field cases, but not in a number of

gnotobiotic and newborn calves (Jarrett, 1987). Discussion following the CIBA Foundation Symposium on Papillomaviruses (CIBA Foundation Synposium 120, p133, 1986) volunteered the information that HPV-6 DNA had been found in the lymphocytes of a patient with laryngeal papillomas (Steinberg) and in one patient with EV (L. Gissmann on behalf of A.J. Faras) but not in a further five EV patients (G.Orth on behalf of S. Obalek) while HPV-5 had been found in the sperm of both an adult male with EV and his unaffected young son (M.S. Campo). Further work on these aspects is required.

Thus, two models of naturally occurring malignancies are associated with BPV :-

1. Bladder carcinomas in which BPV-2 is present in a multicopy episomal state, either as an opportunistic pathogen or exerting its full oncogenic potential in a specialised environment.
2. Alimentary carcinomas, in which BPV-4 appears to play an initiating role, but is no longer required thereafter.

In both systems the involvement of an environmental carcinogenic and immunosuppressive agent is central to malignant progression. All of these elements have counterparts in the human situation.

3.52 Genital cancers and HPV

At the same time as HPV DNA was detected in dysplastic lesions, viral components were also sought in malignant tumours. Neither PV particles nor viral antigen were observed in genital carcinomas thought to have developed from papillomas (Gissmann, 1984; Syrjanen, 1984) but PV DNA could be readily detected in blots or on sections using swabs, scrapes or biopsies in up to 90% of malignant cervical disease (Singer and McCance, 1985) with HPV-16 in about 50% of cases, HPV-18 in about 20% and others (HPV-10, 11, 31, 33 and 35 and other unidentified types) in a small proportion of biopsies (Gissmann and Schwarz, 1986).

The massive prospective study in Finland begun in 1981 and involving more than 500 women in six-monthly follow-ups, (Syrjanen, 1983a; Syrjanen et al., 1985b) has shown that progression was most frequent (33.3%) and regression most infrequent (5.6%) in HPV-16 lesions (Syrjanen et al., 1987a). On the other hand, a number of HPV-6 and HPV-11 containing lesions also progressed during the follow-up. Furthermore HPV-6 has been found in one of a sample of 29 cervical carcinomas examined by Gissmann and colleagues (1983) as well as in locally invasive Buschke-Lowenstein tumours (Gissmann et al., 1982) and it would be premature to dismiss as insignificant the oncogenic potential of the HPV types most commonly associated with condylomata acuminata.

Recently an increasing number of cases of infection with multiple HPV types has been reported. McCance et al. (1985b) described five women with multifocal genital intraepithelial neoplasia in each of whom HPV-6 and HPV-16 was found; Winkler et al. (1986) described both HPV6 and HPV-16 in 65% of 43 patients with similar lesions; Del Mistro et al. (1987) identified two types of HPV in 13 of 15 males with uretheral condylomata acuminata and it could be that HPV types act synergistically - possibly with types 6 and 11 increasing cellular proliferation such that an increased incidence of AMF is induced by HPV-16.

One of the most worrying aspects clinically of cervical carcinoma development today is the rapidity with which some lesions progress. Paterson et al. (1984) found 56 women out of 312 with cervical cancer had had negative smears in the three years preceding cancer development. When the smears were reviewed, about half were regraded as abnormal, but in the others, a short pre-invasive phase seems likely. This rapid progression was more common in younger women. Syrjanen et al. (1985a) reported a well documented case of a lesion containing HPV 16/18 progressing to invasive cancer in less than three years and a further 5.5% of 343 women who progressed to CIS

within eighteen months. There is increasing evidence that HPV-16 and HPV-18 can be found in the cervix of cytologically normal women. In situ hybridisation of the cells from cervical swabs has shown these types in 5% of the first 300 examined (Wagner et al., 1984). Furthermore in Britain, McCance et al. (1985a) and Murdoch et al. (1988) have shown HPV-16 to be present in age-matched controls with colposcopically and histologically normal cervices as well as in normal tissue from sites adjacent to the HPV-16 containing lesions, a situation similar to the persistence of HPV 6/11 in DNA in normal tissue from sites adjacent to laryngeal papillomas (Steinberg et al., 1983). Indeed, a recent publication using the sensitive polymerase chain reaction (PCR) has shown HPV-16 and/or HPV-11 sequences to be present in 70% and 84% respectively of two groups of women with cytologically normal cervices (Young et al., 1989; Tidy et al., 1989).

HPV-6 DNA has also been detected in 10% of cervical scrapings from cytologically normal women attending a sexually transmitted disease clinic (Wickenden et al., 1985), viral antigen in 2 out of 25 histologically normal cervical epithelium (Walker et al., 1983) and even virus particles in 14/22 cervical biopsies with no evidence of HPV infection (Syrjanen et al., 1985b). Thus HPV can replicate without producing clinical symptoms and could be transmitted by asymptomatic carriers.

The suggestion that HPV of some types at least is capable of remaining in a latent state is further supported by the much higher frequency of HPV-16 and HPV-18 positive smears in pregnant women, perhaps due to reactivation under different immunological and hormonal influences (Gissmann and Schwarz, 1986). The occurrence and regression of flat lesions of Bowenoid papulosis of the anogenital skin is similarly associated with pregnancy and delivery (Pfister, 1987b).

It has already been pointed out that in the male, HPV-16 can replicate to a limited extent and produce small amounts of infectious virus (Gissmann and Schwarz, 1986). It could be that in cervical cells,

replication is inhibited by tissue specificity, with persistence occurring more readily than in the male, allowing time for a variety of co-factors to exert synergistic effects. In this sense, the HPV-16-papilloma-carcinoma progression may be compared to the BPV-2 model where virus is produced in specific cutaneous lesions, but when it reaches the bladder epithelium, perhaps from the paragenital lesions with which BPV-2 can also be associated (Jarrett, 1985), it can persist and undergo limited transcription but nothing more. The difference between the two systems, however, lies in the episomal persistence of the BPV-2 genomes in bladder carcinomas compared with the integrated pattern almost invariably seen with HPV-16 in cervical carcinomas (Boshart et al., 1984; Durst et al., 1985, Shirasawa et al., 1986, Murdoch et al., 1988).

Two distinct patterns of integration of HPV-16 have been observed either in head-to-tail arrangements or as a single copy, but integration occurs at a remarkably specific point on the viral genome usually between E1 and E2, such that only E6, E7 and part of E1 can be transcribed from the viral early promoter (Gissman and Schwarz, 1986). Occasionally integration occurs slightly downstream within E2 or even L2 (McCance 1986) and Shirasawa et al., (1987) recently reported a cell line in which the complete HPV-16 genome was integrated. Transcription, predominantly of E6 and E7, in cervical carcinomas and cell lines derived from them, has already been described (Section 2.32). While the function of these gene products continues to be investigated in transformed cell lines, their exact role in-vivo is unknown, and indeed transcription of early genes is not always found in biopsy specimens of cervical carcinomas (Lehn et al., 1985; Orth, 1986b; Pater and Pater, 1988). This could be due to inactivation of transcription or to a very low copy number of HPV-16 DNA per cell (Orth, 1986b). Perhaps in these few tumours a situation exists more akin to that of BPV-4 induced tumours in which PV-DNA is no longer detectable in the alimentary canal carcinomas. One might speculate

that human cervical carcinomas being removed as expediently as possible for the patient's welfare have rarely reached this final stage of oncogenic potential.

Nevertheless, HPV specific mRNAs are found in over 80% of cervical tumours (Orth, quoted by McCance, 1986) and indeed it has been suggested that there may be unregulated persistent E6/E7 transcription due to the disruption on integration of E2, with its trans-activating and counter-acting repressor functions (Cripe et al., 1987). These workers suggested that keratinoytes contained a cellular factor which acted on the short enhancer element 5' to E6 and E7 and which would normally be suppressed by a functional E2.

The integration of HPV-16 and HPV-18 at random sites on the cellular genome would suggest that the site of integration is not important (McCance, 1986). However some particular cellular insertion sites may alter the expression of cellular genes. Thus in an analysis of four cervical carcinoma cell lines and one primary cervical cancer, Durst et al., (1987) found integration near and activation of c-myc in HeLa and C-4I cells while in the other two lines no close linkage to cellular oncogenes was found. The HPV-16 sequences localised to two areas which contained the proto-oncogenes c-src-1 and c-ras-1. Similarly, Shirasawa et al. (1987) in 3 of 5 Japanese derived cervical carcinoma cell lines detected c-myc and c-Ha-ras expression at about nine times the level found in normal cells. Certainly transfection studies with HPV-16 DNA in vitro have shown that co-operation between the viral DNA and EJ-ras can occur to establish transformation (Matlashewski et al., 1987). In a study of invasive cervical cancers, Riou et al. (1985) found a 3-30 fold amplification of c-myc and/or c-Ha-ras in 21/37 advanced tumours. The absence of oncogene amplification in the earlier stage tumours suggested that a role for myc and ras was confined to the late stages.

3.53 The rabbit papilloma-carcinoma complex

The oldest model of viral cutaneous carcinogenesis is that of the Shope papilloma-carcinoma complex. The cotton tail rabbits of mid-West America appear to be "horned" (Figure 6) due to papillomatous growths which may persist, regress or undergo malignant transformation in about 25% of cases. In 1933 Shope and Hurst discovered that the filterable agent from these papillomas could induce papillomas in domestic rabbits, but could not then be transmitted from domestic rabbits, which showed a much higher incidence of progression to carcinoma. Yet, the gross and histological appearances of the tumours were identical and even at this time Shope noted that perhaps they represented the two extremes of a neoplastic process. Viral antigen was abundant in superficial cells of the cottontail papillomas, but not in domestic rabbit papillomas (Noyes and Mellors, 1957). Nevertheless, Ito and Evans (1961) showed that DNA extracted from domestic rabbit papillomas was still infectious and tumorigenic and could be detected in granular cell nuclei (Orth et al., 1971) by in situ RNA-DNA hybridisation.

More recently, Stevens and Wettstein (1979) showed that the DNA isolated from papillomas, primary carcinomas and even metastatic carcinomas all contained equivalent amounts of viral DNA (10-100 copies per diploid cell equivalent of DNA). The DNA appeared almost entirely episomal but variable in amount and length (Wettstein and Stevens, 1982). Integration into the host genome was rare, occurring in only a few primary tumours, but also in the Vx2 and Vx7 transplantable tumours (Sugawara et al., 1983) which had been induced by CRPV 50 years earlier and maintained in vivo by serial passage.

That the CRPV genome participates in the malignant process was shown by early work comprehensively reviewed by Kreider and Bartlett (1981), but the relationship between persistence of the viral DNA and progression is far from clear. Wettstein and Stevens (1981) showed that a small amount of viral RNA was transcribed in a

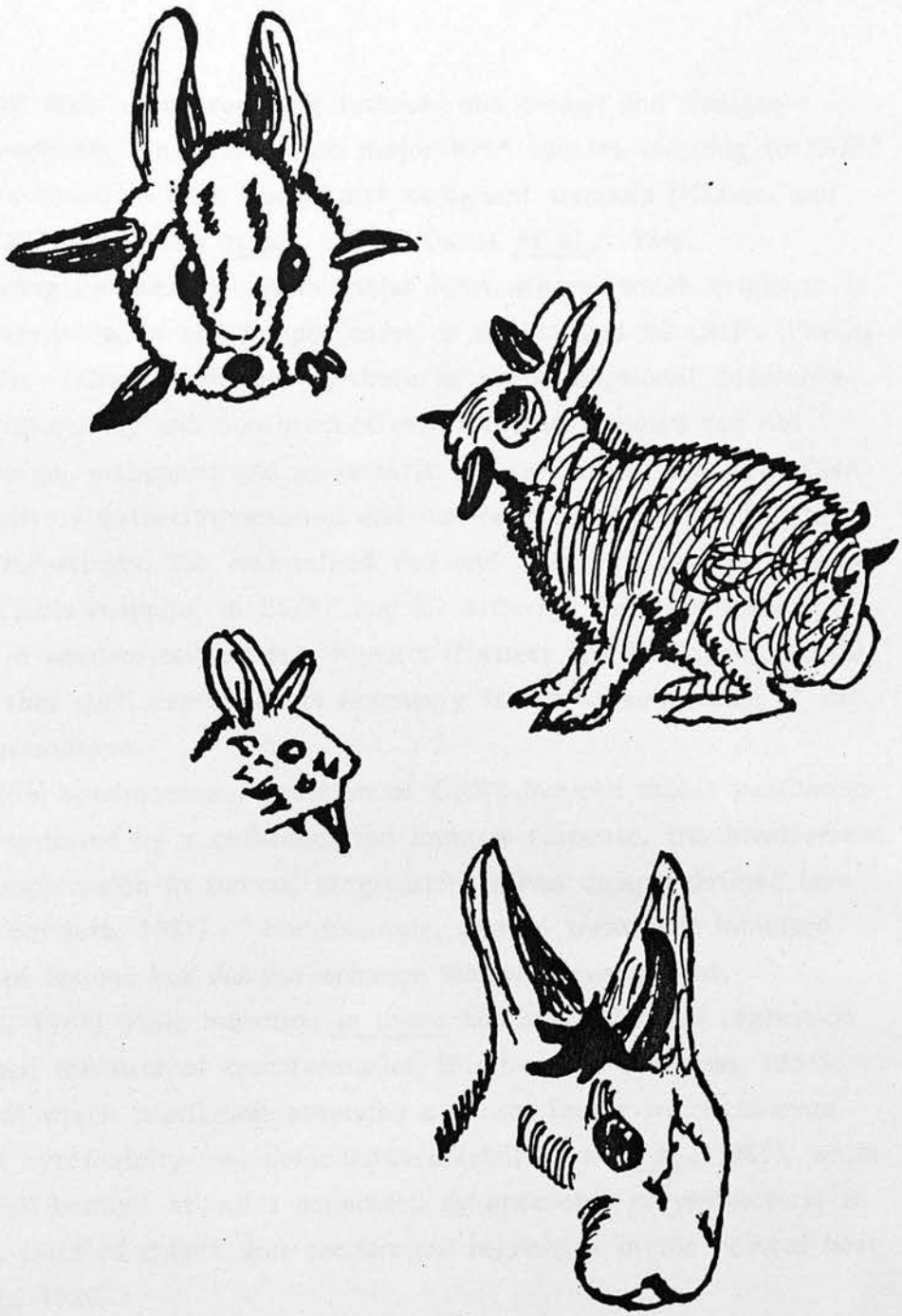


Fig 6. "Horned cottontail rabbits infected naturally with CRPV (drawn by Alison F. Cubie after sketches of Ernest Thompson Seton reproduced in Kreider and Bartlett, 1981).

proportion of both virus-producing tumours and benign and malignant non-virus-producing tumours. Two major RNA species mapping to E6/E7 and E2 were found in both benign and malignant tumours (Nasseri and Wettstein, 1984a; Phelps et al., 1985; Danos et al., 1986). Virus-producing tumours also have major RNA species which originate in E1 of the early region and include most of the L1 and L2 ORFs (Phelps et al., 1985). Clearly, therefore, there is a transcriptional difference between productively and non-productively infected tumours but not between benign, malignant and metastatic lesions despite the viral DNA being exclusively extrachromosomal and non-rearranged (Phelps et al., 1985). Interestingly, the maintained Vx2 and Vx7 tumours also produce RNA transcripts mapping to E6/E7 and E2 although they are fully integrated in head-to-tail tandem repeats (Nasseri and Wettstein, 1984b), suggesting that such expression is necessary for the maintenance of the malignant phenotype.

While spontaneous regression of CRPV-induced rabbit papillomas is clearly mediated by a cell-mediated immune response, the involvement of immunosuppression in tumour progression is less clearly defined (see Kreider & Bartlett, 1981). For example, steroid treatment inhibited regression of lesions but did not enhance tumour development, (McMichael, 1967) while infection in utero not only inhibited regression but increased the rate of transformation (Fischer and Syverton, 1951). In rabbits in which papillomas persisted a serum factor which blocked lymphocyte cytotoxicity was demonstrated (Hellstrom et al., 1969), while the enhanced immune stimulus generated by anaerobic corynebacteria in the normal flora of rabbit skin encouraged regression in the natural host (Seto et al., 1980).

Thus in the CRPV papilloma-carcinoma complex there is an interplay of host factors including genetic background, environmental factors including restricted distribution and the effects of co-carcinogens, coupled with the immune response to virus challenge. CRPV is therefore an exciting and important model for similar human neoplasms.

Unfortunately, research with CRPV has been hampered by the limited supply of virus from infected wild cottontail rabbits and by the lack of availability of syngeneic rabbits for studies at the cellular level. With the supply of CRPV being assured through the successful cloning of the genome (Wettstein and Stevens, 1981) and the development of syngeneic rabbits, it should be feasible to define the immunobiology of progression and spontaneous regression more thoroughly (Kreider and Bartlett, 1985).

3.54 Skin cancers in epidermodysplasia verruciformis

The comparison between the Shope papilloma-carcinoma progression and that seen in EV patients with pityriasis-like warts is striking. The genetic background of the patient, leading to an inherited predisposition to warts, plays a role in the initiation of the neoplastic process, just as the genetic background of rabbits influences the eventual outcome of infection. Although spontaneous regression is a very rare event in EV, it has been observed in a few patients infected with HPV-3 (Jablonska et al., 1982a). Progression is much more common and is associated with a depressed cell mediated immunity, detected by reduced cutaneous responsiveness to DNCB and of lymphocytes to non-specific mitogens (Jablonska et al., 1982b) just as depressed cellular immunity could influence the rabbit papilloma progression as described above. Similarly, as CRPV infection of rabbits in utero or in the neonatal period decreases the regression rate, perhaps too in EV, infection at an immunologically immature stage, as in utero, is responsible for the life-long persistence of EV infections. (Jablonska et al., 1983). The influence of extrinsic factors in both cases is also obvious - the co-carcinogenic effects of tar and methylcholanthrene in the Shope system and the localisation of carcinoma development in HPV-induced lesions to sun exposed areas in EV patients. Ultraviolet irradiation is well recognised to induce transient immunosuppression and to increase viral skin infections (Norval et al., 1986). The carcinogenic effect of X-rays applied to premalignant lesions in EV patients has also been noted,

with resulting deeply invasive carcinomas (Jablonska et al., 1983). These comparisons between the rabbit and EV models of viral oncogenesis are shown in Figure 7.

There are of course some differences in the two systems. For example, CRPV-induced lesions occur characteristically at sites of trauma, whereas EV lesions, although most abundant at exposed and traumatised sites, are disseminated all over the body. CRPV infections are totally confined to hair-bearing epidermis and it has been suggested that the susceptible cell may be the migrating wound epithelium derived from the hair follicle sheath cell (Kreider and Bartlett, 1985). Given the distribution of lesions, this is unlikely to be true for EV. Carcinomas developing from rabbit papillomas are usually invasive with metastases in the regional lymph nodes common and pulmonary metastases occurring in 25% of such rabbits (Kreider and Bartlett, 1981), whereas EV carcinomas are more frequently only locally destructive although occasional metastases have been reported (Ostrow et al., 1982).

At the molecular level, plenty of virus particles can be found in both cottontail papillomas (Stone et al., 1959) and in EV lesions including those that have progressed as far as CIS (Ruiter and van Mullem, 1970), but none are detectable in domestic rabbit papillomas, CRPV-induced carcinomas or EV carcinomas (Orth et al., 1980). The papillomavirus genome, however, is present in all virally-induced tumours in both systems. The rabbit papillomas are all produced by a single virus type and CRPV would appear to have a well conserved genome (Yaniv, 1986) whereas EV lesions are induced by a large number of HPV types. On the other hand, carcinomatous lesions harbour only HPV-5 and closely related types (Ostrow et al., 1982; Pfister et al., 1983; Lutzner et al., 1984; Yutsudo et al., 1985) leading to the suggestion that HPV-5 related types are themselves endowed with malignant potential in the presence of other influences. Certainly malignant conversion does not depend on immune status, since EV patients harbouring only HPV-3 and no cancers have an equally impaired CMI to those with HPV-5 tumours (Glinski et al., 1981).

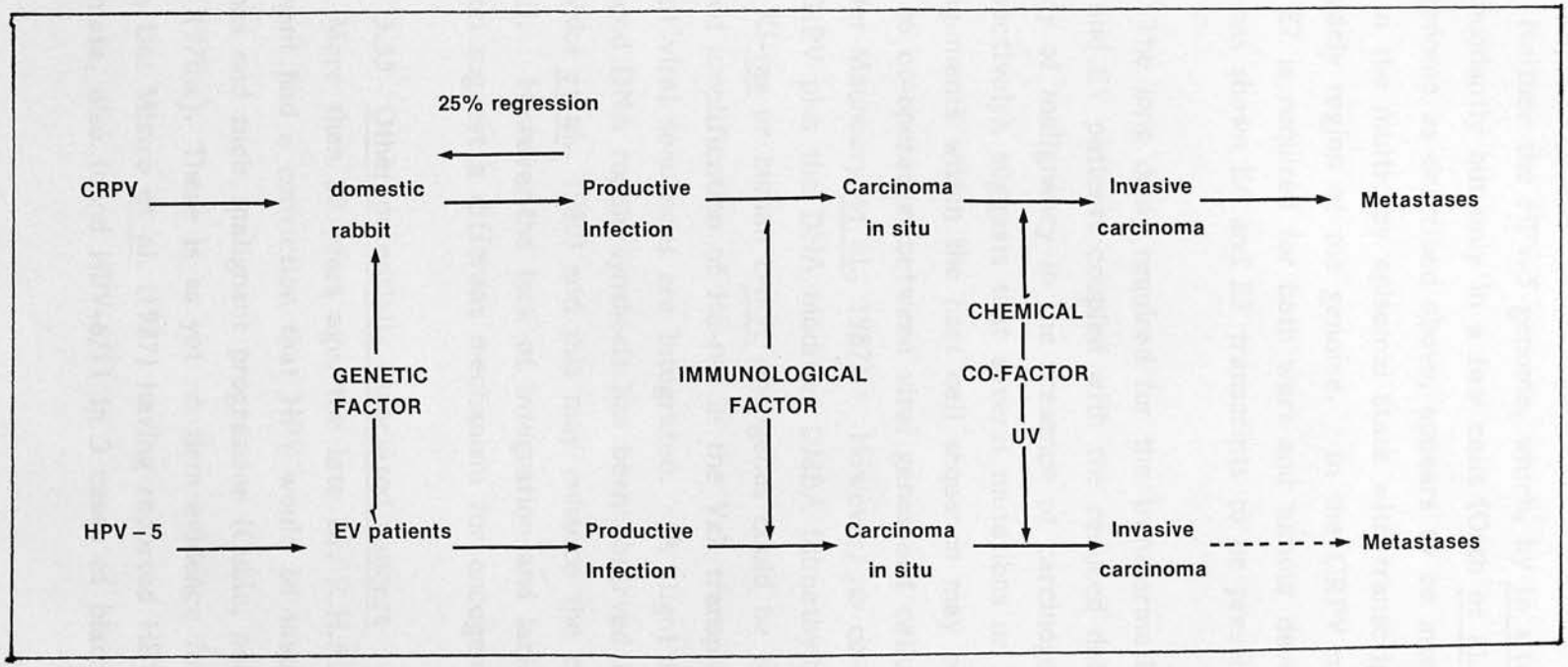


Fig 7

Comparison between the rabbit model of PV carcinogenesis and HPV-5 in EV patients.

Neither the HPV-5 genome, which, by in situ hybridisation was found abundantly but only in a few cells (Orth et al., 1980), nor the CRPV genome as described above, appears to be integrated. Rather they remain in the multicopy episomal state with transcription being restricted to the early region of the genome. In the CRPV model, expression of E6 and E7 is required for both wart and tumour development, and Orth (1986a) has shown E6 and E7 transcripts to be present in EV carcinomas also.

The long delay required for the transformation of warts in both rabbits and EV patients coupled with the reduced delay and increased frequency of malignancy in the presence of carcinogenic agents (tar and UV respectively), suggests that several mutations or DNA re-arrangements within the host cell sequence may be required, perhaps leading to co-operation between viral genes and cellular oncogenes (Schneider-Maunoury et al., 1987). However, no co-operation of CRPV or of CRPV plus the DNA modifier DMBA (dimethylbenzanthrene) with Ha-ras, Ki-ras or human c-myc oncogenes could be found except for a three-fold amplification of Ha-ras in the Vx7 transplantable carcinoma where all viral sequences are integrated. A slight impairment of UV-induced DNA repair synthesis has been observed in some EV patients (Proniewska et al., 1980) and this may enhance the carcinogenic effect of UV itself. However,the lack of integration and lack of oncogene activation suggest a different mechanism for oncogenic progression.

3.55 Other potentially associated cancers

More than 20 years ago, the late Dr. R.H.A. Swain of this Department had a conviction that HPV would be involved in bladder papillomas and their malignant progression (Cubie, personal recollection; Ogilvie, 1970a). There is as yet no firm evidence for such a process although Del Mistro et al. (1987) having reported HPV-6/11 in uretheral condylomata, also found HPV-6/11 in 3 cases of bladder condylomata.

In 1983, Spradbrow and his colleagues in Queensland who were aware of the association of BPV with carcinomas on sun-exposed unprotected skin of sheep and cattle (Vanselow, 1982; Ford et al., 1982), examined four human hyperkeratotic skin cancers attributed to chronic exposure to sunlight and found PV particles in one. More recently, Gassenmaier et al., (1986) in Germany found evidence of HPV in 14/32 cases of solitary keratoacanthoma, most being associated with HPV-25, and Jablonska et al., (1987) reported a new HPV, HPV-36 (related to HPV-5) in a case of actinic keratosis in an immuno-competent individual. Other skin lesions will no doubt continue to be examined in both normal individuals and in the immunocompromised in the search for further HPV involvement.

Although the best known site of HPV infection in the respiratory tract is the larynx, other lesions have been probed for the presence of HPV. Thus while six patients with verrucous carcinoma of the larynx all showed HPV-16 related sequences (Brandsma et al., 1986) only one of 42 biopsies of laryngeal carcinomas harboured HPV (HPV-30; Kahn et al., 1986) and similarly only one of 24 biopsies of carcinoma of the lung of various histological types contained HPV, again HPV-16 (Stremlau et al., 1985. However, a metastatic squamous cell carcinoma of the lung in an adult who had had juvenile laryngeal papillomatosis without radiation therapy or a history of smoking, contained HPV-11 in high copy number (Byrne et al., 1987). Fungiform papillomas of the nose appear to harbour HPV-11 whereas in inverted nasal papillomatosis only 2/13 had detectable HPV sequences (Brandsma et al., 1987).

The oral cavity has also proved a popular site for investigation. In general the papillomatous lesions appear to harbour HPV, usually of HPV-11 related types (Naghashfar et al., 1985; Loning et al., 1985) but so also did leukoplakias (Loning et al., 1985) and, surprisingly, 7/8 lesions of lichen planus harboured HPV-16 related types (Maitland et al., 1987). This HPV-16 variant was also found in 5/12 normal oral tissues and appeared to have the same restriction endonuclease pattern as the HPV-

16 variant found by de Villiers et al. (1985) in a specimen of a tongue carcinoma.

These results probably represent only the tip of the iceberg of the search for HPV sequences in malignant tissues, but they include two interesting findings. Firstly Stremlau's patient who developed a lung carcinoma which contained HPV-16 had had a cervical cancer removed 9 years earlier and it has been known for some time that the incidence of lung cancer in the first five years after treatment for cervical cancer is unusually high (Mould & Barrett, 1976). While the anaplastic nature of the lung carcinoma could not confirm that this was a metastasis, the presence of HPV-16 sequences in pelvic and para-aortic lymph nodes of patients with cervical cancer has been found (Lancaster et al., 1986; Fuchs et al., 1988 and 1989). Secondly, the patient reported by Byrne et al., (1987) in whom a metastatic lung carcinoma could be related to laryngeal papillomas also developed a liver metastasis in which extrachromosomal HPV-11 was found, but with a large 2.2Kb tandem repeat in the LCR of the viral genome. Duplication of transcriptional control and enhancer elements could contribute to carcinogenic progression and it is interesting to note that similar repeats within the LCR have been noted in the cloned HPV-6 DNA from a Buschke-Lowenstein tumour (Boshart and zur Hausen, 1986) and from an unusually aggressive verrucous carcinoma of the vulva (Rando et al., 1986a, b). Watanabe et al. (1984) reported that a BK virus mutant with duplications in the control region for early transcription could transform rat or hamster cells more efficiently than wild type BK giving it a replicative advantage as found with the HPV-6 and HPV-11 variants described above. This interpretation should be viewed cautiously, however, in the light of recent work by Kasher and Roman (1988) in which they studied 7 HPV-6b clones, which, after propagation in E.coli, presented 3 distinct sequence patterns due to insertion and deletions occurring at the same site within the purine-thymidine rich region of the LCR and considered that this was a "hot spot" for recombination in E.coli.

4. Immune Responses to Human Papillomaviruses

Studies with animal PVs have shown that both humoral (HI) and cell mediated immune (CMI) responses develop in papilloma infections (Lee and Olson, 1969) and that regression is due in many cases to a CMI response (Kreider, 1963; Lee and Olson, 1968). Bovine papillomas continue to grow in the presence of circulating antibodies and then regress simultaneously following lymphocyte infiltration. Over the years, many investigators have attempted to clarify the roles of different parts of the immune response in HPV infections.

4.1 Humoral Responses

The first experiments to investigate the frequency with which spontaneous regression occurred in wart virus infections looked at antibody development. Antibody could be demonstrated by various techniques in 45-70% of patients with warts particularly around the time of regression, and was frequently of the IgM class and therefore unlikely to offer protection from subsequent attacks (see Cubie, 1972a). Indeed, Matthews and Shirodaria (1973), using immunofluorescence on wart sections, found HPV-specific IgM antibodies in 100% of patients with regressing warts, but only in half of those whose warts were not regressing. The presence of CF antibodies appeared to Pyrhonen and Johansson (1975) to be associated with rapid cure. Seventy-five per cent of their patients with CF antibodies had lost their warts within 2 months, and it was suggested that the antibodies could be responsible for rejection. However, they also noted that rapid regression occurred in some people in the absence of detectable antibodies and concluded that CMI must be responsible here.

Several workers have used indirect immunofluorescence to look for antibodies to HPV using sections of skin warts as antigen source. Genner (1971) was the first to describe this method but the reactions

obtained were weak. The technique was also used by Matthews and Shirodaria (1973), Pass and Maizel (1973), Shirodaria and Matthews (1975) and by Viac et al. (1977b). In all of these studies the incidence of antibody development was higher in those whose warts were regressing.

When patients were examined according to the clinical type of wart present, approximately 50% of those with plantar warts (generally myrmecia) and 25% of those with hand and other types of wart were found to have antibody by gel diffusion amplified by countercurrent electrophoresis (Cubie, 1972b). Antibody was slow to develop during an infection, could persist for at least 13 years, and did not appear to prevent re-infection. Yet some people with repeated attacks never developed detectable antibody and conversely several people who thought they had never had warts did develop antibody, suggesting sub-clinical infection (Cubie, 1972b). The frequent occurrence of sub-clinical or latent infection was confirmed by Pyrhonen (1978) who found 52% (33/64) of a group of medical students without any history of warts had HPV antibodies by immunodiffusion. He also noted many patients with a weak antibody response and no regression of their lesions during the period of follow-up.

These studies were, of course, carried out using pools of semi-purified virus particles, before the plurality of HPVs was fully recognised and the specificity of each antibody response or of the HPV type causing infection was not known. However, HPVs isolated from different epithelial lesions were shown to be antigenically distinct when no cross-reactions were obtained with animal antisera raised against different HPVs isolated from skin warts (Gissman et al., 1977; Orth et al., 1977; Pass et al., 1977), and so a certain amount of re-evaluation of serological data was carried out using newer serological techniques such as radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Pfister and zur Hausen (1978) describe an RIA using an HPV-1 antigen (or more accurately an HPV 1-3 antigen since the typing was based on protein analysis of pooled plantar lesions) and iodinated anti-

HPV-1 rabbit antiserum. In a nonselected population, maximal prevalence of antibodies to HPV-1 (50%) was found in the age group 11-20 years, this being the age group in which HPV-1 induced warts are most frequently seen. With increasing age, the percentage of HPV-1 antibody positive sera decreased. However, in looking at specific populations with warts and other lesions, 45-55% of adults with plantar and common warts had antibody to HPV-1, as had 53% of 47 patients with condylomata acuminata, approximately 40% of patients with various carcinomas and 26% of patients with Hodgkin's disease. Pyrhonen et al. (1980) used an ELISA test, but again with pooled papilloma homogenates containing several HPV types as antigen, and found IgG antibodies in 44% and IgM in 71% of people with various types but mostly common warts.

Because it just is not possible to produce sufficient virion material from individually typed lesions (other than perhaps for HPV-1 where particle production is high), only very small numbers of sera have been examined using totally type specific reagents. Jablonska et al. (1982b) showed only 3 out of 21 patients with flat warts to have anti-HPV-3 antibodies and 3 out of 12 with common warts to have anti-HPV-2 antibodies. Similarly, Kienzler et al. (1983) report anti-HPV-1 antibodies in 39% of people with myrmecia. In patients with EV, 72.8% had specific antibodies to HPV-1, 2 or 3 using immunodiffusion, whereas 80% of healthy family members of EV patients did not, arguing against a protective role for antibodies (Jablonska et al., 1980). In a study of patients with recalcitrant common warts treated with bleomycin (Bunney et al., 1984) HPV-2 antibodies were found by immunofluorescence on sections of common warts in sera from 8/22 (36%) patients before treatment and in 12/15 (80%) after 6 weeks of treatment with or without HPV-1 antibodies in addition (Bunney and Cubie, unpublished data).

In a very recent large survey in Belfast, using an indirect immunofluorescent test to detect specific IgM antibodies, Steele and

colleagues (1988) found 23.4% to have HPV-1 antibodies and 44.5% to have HPV-2 antibodies in a population of 376 patients with cutaneous warts. None of 50 randomly selected patients had anti-HPV-4 IgM.

The realisation that PVs shared a common antigen, detectable on SDS-disruption of purified particles (Jenson et al., 1980) gave a different approach in providing antigen for serological testing. Using a disrupted BPV-2 antigen, Baird (1983) described an ELISA in which he reported that patients with anogenital warts, CIN, or invasive carcinoma of the cervix, all had higher antibody levels than control groups of children or adults. Positive results in 95%, 60% and 93% of cases respectively were obtained when measured simply by comparison of absorbance readings. However, the absorbancies in all patients were very low (< 0.3) and although some positive responses were noted in a recent study in similar patients, using a similar ELISA system, the very high proportion of positives noted by Baird was not observed (Beiss et al., 1987).

Very recently, attempts have been made to examine the serological response in patients with genital warts and cervical dysplasias using antigens prepared by the expression of fragments of HPV-DNA molecularly cloned in bacteria or by synthetic production of polypeptides determined from the DNA sequences of specific ORFs. By Western blotting with HPV-6b L1 fusion protein as antigen, Li et al. (1987a) found specific antibodies in 18/30 patients attending a colposcopy clinic. In more extensive studies, Jenison et al. (1988) also using Western and dot-blotting and fusion proteins to ORFs of HPV-6b and HPV-16, reported that the predominant activity of patients with condylomata acuminata was to the major capsid protein encoded by HPV-6b L1, although occasional responses to L2, E4 and E2 were also detected. In their hands, sera which reacted with HPV-6b L1 did not react with HPV-16 L1 despite the considerable conservation of this ORF among PVs.

Care must be taken, however, in assessing the specificty of L1 fusion proteins for serological studies. Li et al. (1987a) noted, for

example, that experimentally produced rabbit antiserum to their particular HPV 6b L1 could recognise L1 proteins in both HPV-1 and HPV-6/11 sections. Furthermore, the use of monoclonal antibodies to BPV-L1 has shown the existence of both type-specific and group specific epitopes within L1 (Nakai et al., 1986). Possibly the group-specific epitopes may be dependent on conformation, while Western blots which detect mainly linear epitopes would reveal only type-specific reactions (Jenison et al., 1988). Certainly, work from Strike et al. (1986) showed the group-specific region of L1 to contain beta turns, with a highly hydrophilic region and several well conserved stretches with low polarity, but they too used Western blots in their analysis.

Very recently an HPV-16 L1 fusion protein expressed in vaccinia virus was described (Browne et al., 1988). Antibodies to it and to a synthetic peptide to the C-terminal 14 amino acids of HPV-16 L1 both reacted well with the HPV-16 L1 target in Western blots, whereas group specific polyclonal serum and antiserum to HPV-6b L1 fusion protein failed to react. Furthermore, a monoclonal antibody to the HPV-16 L1 fusion protein has been produced which is capable of detecting antigen in cervical lesions containing HPV-16 or HPV-33 but not HPV-6 or HPV-11. Although one does not expect late antigens to be produced frequently in HPV-associated cervical neoplasia, it might be worth while examining sera from patients with koilocytic lesions and from normal controls for antibodies to HPV-16 L1.

For some serological investigations, the L2 ORF may provide a more satisfactory source of specific antigens since this ORF is not conserved among PVs apart from a short sequence near the N-terminus (Komly et al., 1986). Both Komly and co-workers and Firzlaff et al. (1988) have shown the specificty of antisera to a truncated fusion protein of L2 from which the N-terminus is absent. Alternatively, a 13 amino acid synthetic peptide to the C-terminus of L1 of HPV-6b has been shown to be type specific (Li et al., 1987a), like the HPV-16 L1 synthetic peptide described above (Browne et al., 1988).

4.2 Cellular Immunity

4.21 *In vitro* studies.

The recognition of specific antigens by immune lymphocytes can be measured *in vitro* by three different reaction principles (Kirchner, 1986): the production of biologically active molecules (lymphokines) on specific antigenic recognition by lymphocytes, the proliferation of specific clones of lymphocytes and the cytotoxicity of lymphocytes.

4.211 HPV-specific reactions

HPV-specific reactions can be detected using tests under the first two of these principles. The leucocyte migration inhibition test (LMI) is based on the production and effect of lymphokines and was first used by Morison (1975a) in a study of 118 patients with warts, utilising a semi-purified pooled wart extract as antigen. Most of the patients developed a positive response at or near the time of resolution of their lesions but the response only lasted about 3 months. However, of 9 patients who showed a positive response at the time of first examination, 8 were clear of their warts within 2 months, suggesting that a positive response was an integral part of successful resolution.

The presence of an LMI factor was similarly shown in 4/7 patients who had had warts for less than one year (Lee and Eisinger, 1976). A single case of LMI in response to a specific HPV-3 antigen was reported by Jablonska *et al.* (1982a), in a patient with EV in whom all lesions slowly regressed after two pregnancies. A positive LMI response was detected from 6-18 months after the second delivery.

Lymphoproliferative (LP) responses have been used more frequently as an *in vitro* measure of CMI. Again using a semi-purified virion antigen, Ivanyi and Morison (1976) found "mild" stimulation in patients with active warts was followed by increased stimulation after resolution. Similarly, 5 of the 7 patients who had warts of short duration in Lee and Eisinger's study (1976) gave positive responses after

6 days of culture although the extent of proliferation was low. Nevertheless, all these results from both LMI and LP studies support the concept that a cell-directed immune response plays a role in regression of human warts.

However, Lee and Eisinger (1976) also detected positive responses in a group of people who thought they had never had warts, again giving credence to the idea of latent infection, and a further group whose warts had lasted a long time showed relatively little response. Because the response to non-specific mitogens such as phytohaemagglutinin (PHA) is normal in this group of patients, it was suggested by these workers that there might be a group of people genetically unresponsive to virus, thus enabling persistence to occur or tolerance to the virus to be induced, perhaps by immunological blocking of lymphocytotoxic cells as suggested in persistent rabbit papillomas (Hellstrom et al., 1969). A soluble factor of this type was apparently found in the wart tissues of a patient with warts of 20 years duration - the addition of a saline extract of one of the patient's warts abolished a positive LMI response to HPV antigen (Freed and Eyres, 1979).

4.212 Non-specific reactions

It was recognised many years ago that immunosuppressed patients such as renal allograft recipients (RAR) and patients with deficiencies of CMI secondary to Hodgkin's lymphoma and chronic lymphocytic leukaemia were more prone to papilloma infections which could become disseminated and non-regressing (Spencer and Andersen, 1970; Morison, 1975b). Several investigators have therefore looked at non-specific CMI responses and peripheral blood lymphocytes for evidence of deficiencies in wart-bearing patients.

Morison (1975c) suggested that wart patients were less responsive than controls to PHA and purified protein derivative (PPD) in LMI tests and the deficiency was related to the duration of the wart infection. Using a sheep red cell rosette assay to measure T-cell levels in

peripheral blood, Chretien et al. (1978) noted decreased levels in 72 healthy people with current warts and 21 who had had warts in the past compared with controls with no past history of warts.

Using specific T-cell markers recently, Lodi and colleagues (1987) noted a decrease in peripheral blood $CD4^+$ cells in patients with both common and genital warts, and a resultant drop in $CD4^+/CD8^+$ ratio. A similar change in ratio in patients with flat warts was due rather to an increase in $CD8^+$ while patients with plantar warts showed no difference from controls. These results reflect the earlier in vitro results obtained by Obalek and his colleagues in Warsaw (1980). PHA-induced LP was not significantly altered in patients with HPV-1 induced plantar warts, but it was reduced in those with HPV-2 and HPV-3 lesions. Jablonska et al. (1980) considered indeed that defects in CMI facilitated infection with HPV-2 and HPV-3. Similarly in 16 women with recalcitrant genital warts, Seski et al. (1978) found a reduction in the LP response to PHA, pokeweed mitogen (PWM) and concanavalin A (Con A), while Avgerinou et al., (1986) reported a reduction in LMI response to tuberculin and DNCB in 30 patients with recurrent genital warts. On the other hand, in small groups of women with genital warts or cervical dysplasia, neither Jablonska et al., (1982b) nor Neill (1984) working in this laboratory, found significant alterations in LP responses to non-specific mitogens.

Most patients with EV show a significant reduction in the LP response to PHA (Prawer et al., 1977) and a decrease in circulating T cells (Jablonska et al., 1980). The deficiencies, however, were similar to those observed in patients with long standing and widespread plane warts, and they were also similar in EV patients whether the infecting type was HPV-3 or the potentially more oncogenic HPV-5 suggesting that the virus itself rather than the extent of T cell defect is a decisive factor in malignant transformation (Jablonska et al., 1982b).

4.213 Cytotoxic cell responses

Killing of virally-infected cells may be mediated directly by natural killer (NK) cells or by antibody dependent cytotoxic cells (ADCC) via antibody bound to target cells. These two functions may even be provided by the same effector cell acting through different receptors (Roitt et al., 1985). One or two attempts to look at this aspect of immunity in vitro have been reported. Neill and Norval (1984) in a study of 105 patients with CIN of varying degrees found no difference in NK activity against Chang liver cells or against the K562 myeloid cell line in any of the patient groups nor in K cell activity in an ADCC assay on Chang cells. Likewise, normal NK activity against K562 cells has been shown in patients with EV (Androphy et al., 1984) although Kaminski et al., (1985) reported heightened NK activity in 4 of 6 EV patients who had multiple skin cancers. On the whole, decreased NK activity is associated with a high incidence of malignancy (Hanna, 1985) and so this result in EV patients is somewhat surprising. However, it is postulated that the increased activity may prevent uncontrolled growth of the malignant skin tumours and explain why metastases are so rare in EV patients (Kaminski et al., 1985). Extending the assay to further groups of patients including some with Bowenoid papulosis and some with HPV-16 associated anogenital carcinomas, Malejczyk et al., (1987) found NK activity against K562 cells to be similar in these patients and in controls.

Until recently it has been impossible to produce HPV-infected cells in vitro to act as specific target cells in cytotoxic assays. However, the establishment of a continuous keratinocyte cell line from an HPV-16 associated vulvar Bowenoid papule (SKV-1 cells) enabled Malejczyk et al., (1987) to look for specific reactions. They found that both the patients with Bowenoid papulosis and those with anogenital carcinoma had markedly depressed NK activity compared with controls. Since different clones of NK cells have been shown to have different target specificities (Herberman and Ortaldo, 1981), it is possible that a

single subset of effector cells mediates the specific decrease in NK activity against HPV-16 containing target cells. The degree of suppression appeared to be related to the degree of malignancy and it is postulated that the inability to eliminate cells infected with potentially oncogenic HPVs might be an important factor in the development of HPV-induced neoplasm. Furthermore, the defective NK activity shown in patients with various malignancies including leukaemias might explain the persistence and progression of HPV-induced lesions in these patients.

Recently, further extensions to this work have been accomplished (Jablonska, 1988; personal communication). EV patients with lesions induced by EV specific HPV types had normal activity against K562 and SKV-1 cells, but three had reduced activity to keratinocytic target cells derived from a premalignant lesion of an EV patient. EV patients with HPV-3 induced lesions showed reduced activity to all target cells tested, in line with their deficient CMI when measured by other parameters. Similarly, only patients with HPV-16 lesions and not those with HPV-6 associated lesions had reduced NK activity towards the HPV-16 containing cell line (Malejczyk et al., 1989). In addition, sera from most of the Bowen's papulosis and carcinoma patients could exert an inhibitory effect on the cytotoxicity of normal NK cells against SKV-1 targets. This further supports the theory already described (Hellstrom et al., 1969; Lee and Eisinger, 1976; Freed and Eyres, 1979) that a serum factor capable of blocking lymphocytotoxic cells accounts at least in part for the persistence and/or progression of papilloma infection.

A different type of cell mediated tumour cell destruction has been observed in vitro by Tagami et al. (1985) in spontaneously regressing plane warts. Primary explant cultures of inflamed warts were shown to contain not only wart-derived keratinocytes but in 9 out of 10 cases, massive numbers of lymphocytes which migrated out of the explants, attacked the keratinocytes and induced degenerative changes. Outgrowths from explants of non-inflamed warts showed keratinocytic monolayers within 10 days as with normal skin, and no

degenerative changes. Although subtype monoclonals were not available at the beginning of the study, cells in a few explants were typed and it was found that suppressor/cytotoxic T cells outnumbered helper cells.

4.22 In vivo Studies

Cell mediated immunity can also be investigated in vivo by looking at the delayed hypersensitivity (DH) skin reactivity to intradermal inoculation of both non-specific and specific viral antigens. Thus, in 1974, Brodersen et al. reported that the tuberculin reaction in a group of previously BCG vaccinated children with common warts was significantly reduced compared with a group of controls, in general agreement with Morison's (1975c) in vitro data of reduced LP to mitogens in wart patients. This lent support to the generally held view at the time that CMI was impaired in patients with warts. Thivolet et al. (1977) reported the use of formaldehyde-inactivated purified HPV as stimulating antigen for intradermal testing and found 75% of patients with a past history of warts reacted positively. In extending these results, the same workers showed that DH reactions were most frequent in patients with warts of 6-24 months duration, developed around the time of regression and lasted longer than detectable circulating antibodies (Viac et al., 1977a). Responses were lowest in a group of patients with flat warts and although only 7 patients were studied, this too mirrors the results of in vitro studies in similar patients (Obalek et al., 1980). In a group of genital wart patients, however, positive responses were obtained as often as in those with plantar warts (Viac et al., 1978).

Repeated intradermal testing with the same antigen was carried out on a group of patients with multiple warts (Viac et al., 1977b). After three such testings, the patients could be divided into those whose warts had resolved following the acquisition of specific CMI and of circulating IgG antibodies (10 patients) and those who showed only weak or non-existent specific immune responses and in whom warts persisted (12 patients). Thus the outcome of similar HPV infections in different

individuals is influenced by their different immune responses to the virus.

4.3 Local Immune Reactions

4.31 Nature of antigens produced

As wart virus infections probably occur wholly within the epidermal element of the skin, access to the systemic immune system is limited and it is appropriate to look for evidence of local immune reactions to the infection.

Using cryostat sections of different skin wart biopsies, intranuclear viral antigen staining is readily observable by fluorescence with hyperimmune rabbit antisera against viruses from similar lesions (Genner, 1971; Pass and Maizel, 1973). Type-specific guinea pig antisera have also been used to detect nuclear viral antigens in HPV-1, 2, 3 and 5 induced lesions using the more sensitive anti-complementary immunofluorescence test (Chorzelski et al., 1983). However, in addition to the viral antigens, Pass et al. (1971) demonstrated cellular antigens of human wart tissue which were present in normal tissue and markedly increased in warts and some epidermal tumours. Using antisera against wart tissue homogenates Pass and Marcus (1973) showed that there were two such nonviral antigens, one nuclear and one on the cell surface. Shirodaria and Matthews (1975) similarly noted that the sera of some patients contained, in addition to antiviral antibodies, an antibody of the IgM class directed against the wart cell itself and present more frequently than the antiviral antibodies.

4.32 Immunocytochemical analysis

4.321 Initial studies

The most obvious form of local immune reaction to wart infection is seen histologically in the massive lymphocyte infiltration

which precedes resolution of a crop of plane warts (Tagami et al., 1974; Tagami et al., 1977; Berman and Winkelmann, 1977). The mononuclear cell-associated injury to the epidermis is similar to that seen in DH reactions and is observed in the patients as an acute inflammatory reaction. The sudden eruption of large numbers of tiny new warts often signals the onset of involution (Berman and Berman, 1978), perhaps because cellular destruction releases infectious particles capable of initiating new infections just prior to the resolution of them all, or because heightened immunological activity results in the expression of previous subclinical infections. Cellular infiltration can also be observed histologically in common warts at the time of regression, but the effects are not so dramatic, oedema and inflammation are less obvious and resolution occurs over a longer period (Berman and Winkelmann, 1980).

In studying regressing plane warts at the ultrastructural level Oguchi et al. (1981a) noted both lymphocytes and activated macrophages in the infiltrate, the former with pseudopodia often extending into epidermal cells and the latter interacting with degenerating epithelial cells, fusing with or invading them. In addition epidermal Langerhans cells showed enhanced activity with increased cell granules (Oguchi et al., 1981b). Specific histochemical stains such as acid naphthylacetate esterase (ANAE) were also used to differentiate some of the cells in the infiltrate, enabling Tagami et al. (1985) to show that lymphocytes, mainly T cells, predominated over macrophages in regressing plane warts and were capable of attacking keratinocytes to bring about degenerative changes.

Staining with ANAE was also used by Syrjanen (1983b) to look at sections of cervical dysplasias. He found increasing numbers of T lymphocytes associated with advancing atypia both in HPV lesions and in lesions lacking koilocytes, and a decrease in the number of B cells as the severity of the lesions increased. No significant differences in T cells, B cells and macrophages were observed amongst

lesions which regressed, remained stationary or progressed (Syrjanen et al., 1984). However, the patterns obtained with this stain are difficult to interpret and the method has been totally superceded by the use of sensitive and specific monoclonal antibodies for subset analysis. With such powerful tools valuable information has been obtained, and it is relevant at this point to describe current understanding of the different elements involved in immune reactions in the skin.

4.322 The skin immune system

The skin immune system comprises many elements, of which the principal ones are the Langerhans cell (LC), a bone-marrow derived dendritic cell of the monocyte-macrophage series with antigen-presenting properties (Stingl et al., 1978), keratinocytes and epidermotropic T lymphocyte subpopulations. Other immunologically relevant cells in the skin include mast cells, macrophages, granulocytes, indeterminate cells, veiled cells and the endothelial cells of capillaries and afferent lymphatics (Bos and Kapsenberg, 1986).

Langerhans cells are the epidermal equivalent of macrophages and as such are central to the skin immune system. They are capable of phagocytosis and of migration from skin to lymph nodes and they play an important role in the induction of many types of T cell response including the induction of proliferative and cytotoxic T cell responses (Katz et al., 1985). LC also play a major role in DH reactions (Toews et al., 1980) in preventing the induction of unresponsiveness to antigen presented cutaneously. This was shown in mice whose first exposure to DNFB occurred through skin depleted of LC, and which, on subsequent challenge onto normal skin, were unable to become specifically sensitised. The mice had become profoundly unresponsive. Thus LC in the skin act as an immune surveillance system, and their absence will lead to tolerance and in the case of viruses, persistence.

Langerhans cells can be identified immunocytochemically by their ATPase activity (Wolff and Winkelmann, 1967), by their expression of major histocompatibility (MHC) Class II antigen (Streilein and Bergstrasser, 1980), their Fc-IgG and C3b receptors (Stingl et al., 1977) common thymocyte antigen (T6; Murphy and Harrist, 1982) and the presence of S100 protein (Cocchia et al., 1981). ATPase activity and the presence of S100 protein can be estimated on formalin-fixed paraffin embedded sections, but the other markers are detected on frozen sections with the appropriate monoclonal antibodies. The MHC Class II antigens are encoded by the HLAD region on chromosome 6 and gene products with specificities DP, DQ and DR have been found within this complex (Roitt, et al., 1985). HLA-DR is the most abundantly expressed surface marker on LC, and activation of the cell results in its increased expression (Berman et al., 1985).

An increase in Langerhans cells in benign skin lesions was observed by Gatter et al. (1984b) and a reduction in number with changes to a stunted morphology in malignant lesions. The majority of cells surrounding the tumours were of the helper/inducer subset (Th/i). At the time it was noted that two cases of verruca vulgaris were an exception to this rule in behaving more like malignant tumours.

In normal skin, HLA-DR expression is restricted to LC, but there is now considerable evidence to show that keratinocytes can be induced to express this antigen in a variety of skin disorders (Aiba and Tagami, 1984; Aubock et al., 1986), particularly those in which there is evidence of lymphocyte infiltration and keratinocyte damage (Lampert, 1984) and including contact dermatitis (Gawkrodger et al., 1987). It is interesting that HLA-DR expression can be induced in neonatal and normal skin by γ-interferon (γ-IFN; Berman et al., 1985) and that weak antigen expression is occasionally noted in normal skin (Gawkrodger et al., 1987).

The interplay of cells and soluble factors is far from resolved, but it appears that activated LC either locally or after migration

to nearby lymph nodes produce IL-1 which attracts T cells from the peripheral circulation and stimulates their proliferation. They in turn infiltrate the dermis and epidermis, producing γ-IFN which leads to the synthesis and expression of HLA-DR on keratinocytes in the immediate vicinity of these T cells. The keratinocytes in response produce epidermal thymocyte activating factor (ETAF) which may further stimulate T cells (Sauder, 1985). Keratinocytes expressing HLA-DR do not appear to have an inductive role since they are apparently never seen in the absence of, or preceding, a dermal infiltrate (Aubock et al., 1986). On the other hand, the stratum corneum can act as a reservoir for preformed ETAF, perhaps enabling it to initiate the earliest events associated with the inflammatory response (Daynes et al., 1985). Finally, it has been postulated that keratinocytes expressing HLA-DR may acquire the ability to present processed antigen in association with DR, and then be recognised as nonautologous by cytotoxic T cells (Morhenn et al., 1985).

4.323 Subset analysis

Iwatsuki and his colleagues in Japan (Iwatsuki et al., 1986) looked further at regressing plane warts and found that LC were present in both the cells of the dermis and the epidermis, sometimes in apposition to T cells. Of the T cell infiltrate, Th/i outnumbered suppressor/cytotoxic T cells (Ts/c). Only about 40% of the infiltrate at the early stages of regression consisted of macrophages and there were a small number of NK cells in 3 out of 4 late inflammatory lesions. Although satellite cell necrosis as a marker of direct cell killing and observable as a damaged keratinocyte surrounded by lymphocytes was observed occasionally, the relative lack of Ts/c cells suggested to these workers that the immune reaction involved was more complex than direct cytotoxicity and probably involved soluble factors such as interleukins and interferon (Iwatsuki et al., 1986).

Gatter et al. (1984b) reported a predominance of Th/i in two

cases of cutaneous warts, but in a large study of 75 nonregressing warts, Chardonnet et al. (1986) found that no T cell subset predominated in cutaneous or mucosal warts induced by HPV-1 or HPV-2, although $CD8^+$ cells were more frequent in lesions with detectable viral antigen. The epidermis was depleted of LC, again particularly in lesions with antigen, but larger numbers of LC were found in the dermis suggesting migration following activation. Overall, 65% of biopsies had an abnormal distribution of LC and 29% had no epidermal LC.

Subset analysis has been more extensively studied in cervical dysplasias with and without evidence of HPV infection than in other cutaneous wart lesions. Morris et al. (1983) made use of some of the first available monoclonals for such analysis and found that in patients with wart virus infection (without evidence of CIN) there was an overall depletion of T cells, and those that were present were predominantly Ts/c. B cells were few in number and LCs were reduced, with those remaining confined to the basal layers having lost their interdigitating processes. By contrast, 3 patients with CIN showed a heavy infiltration of Ts/c cells and an increase in LC with spindle shaped morphology but still lacking cytoplasmic processes. The results suggested that the ability of wart virus to persist in cervical epithelium was a direct consequence of its ability to deplete the tissue of LC and T lymphocytes (Morris et al., 1983). Syrjanen's contemporary study (Syrjanen et al., 1984) showed a slight but not statistically significant decrease in the ratio of Th/i to Ts/c in progressing lesions and recorded, but did not comment on, a large drop in the percentage of LC in progressing lesions.

In contrast to the results from Morris's group, the recent studies of Tay et al. (1987a and b) have shown the ratio of $CD4^+$ to $CD8^+$ to decrease not only in HPV infections but also in CIN, due to a depletion of $CD4^+$ rather than an increase in $CD8^+$ (Tay et al., 1987b). Activated lymphocytes (Tac +) were not observed and there were no significant changes in total T cells or their proportions in the epidermis.

This lack of stimulation or of subepithelial disturbance of T cells could be the result of LC depletion in both HPV and CIN (Tay et al., 1987a) and, together with the selective depletion of $CD4^+$ in the epidermis, suggests a local intraepithelial immune deficiency is associated with HPV and CIN.

In a recent study from our own laboratory, Hughes et al. (1988) also reported a reduction in LC in all biopsies with HPV infection and/or CIN, although in CIN I and CIN II without koilocytes, the reduction did not reach significance. By analysing the differential expression of HLA-D region gene products on LC, they found that HLA-DR positive LC cells were reduced in all groups, but especially in CIN III. HLA-DP positive LCs were also reduced with the reduction being greatest in lesions showing koilocytosis alone, but HLA-DQ positive LCs were increased relative to normal in mild CIN lesions. Given that HLA-DQ expression may be involved in the generation of a suppressor T cell response (Navarette et al., 1985) this is an interesting finding. However, Drijkoningen et al. (1988) looking at 50 warts of hands, feet and genitals, found HLA-DQ epidermal LCs in similar numbers to those expressing HLA-DR, but with fewer HLA-DQ positive cells in the dermis. Further elucidation of the functional significance of the differential expression of different Class II antigens will be required before conclusions can be drawn.

Using a range of markers for LC, Tay et al. (1987a) observed that several subpopulations of LCs were present in cervical epithelium and, in both WVI and CIN, a specific subset identified by the use of antibody to S-100 cytoplasmic protein was selectively and almost totally depleted. Similarly, McArdle and Muller (1986) recorded a decrease in LC in cervical WVI using anti S-100 on archival cervical biopsies, although their finding of increased S-100 activity and therefore LC numbers in CIN alone is in closer agreement with the earlier results of Morris et al. (1983). A very recent report from Barton and colleagues (1988) confirms the reduction of LC in patients with CIN + HPV using

both OKT6 and S-100 markers, but a total depletion of S-100 positive cells as suggested by Tay et al. (1987a) was not noted.

Considering the presence of other cells of the immune system, Tay et al. (1987c) noted NK cells to be present in a few controls but in more than three quarters of biopsies showing CIN with or without HPV. The frequency and pattern of NK distribution was similar in all grades of CIN and the numbers of NK present were small and confined to the stroma. From these results, it was concluded that NK cells were unlikely to be primarily involved in the lysis of infected cells at the tumour site, but they might play a role in the prevention of stromal invasion. Similarly, in looking at macrophage density and distribution, the same workers concluded that although macrophage numbers were increased in CIN $\pm$ HPV, they were not the APCs responsible for induction of the immune response (Tay et al., 1987d).

The immunocytochemical results in WVI described above are summarised in Table 6, from which it can be seen that, in general, HPV is associated with a reduction in LC creating a locally immunodeficient state. T cells may be depleted or increased, but often an increase in $CD8^+$ is evident. Much remains to be elucidated, however, concerning the interplay of other cells. In particular, the expression of HLA-DR on keratinocytes which do not normally express this antigen, has been reported in regressing plane warts (Aiba and Tagami, 1984), in common warts (Aubock et al., 1986) and in cervical WVI (Morris et al., 1983), although both Gatter et al. (1984a) and Chardonnet et al. (1986) failed to detect HLA-DR staining in various cutaneous wart lesions.

The involvement of activated keratinocytes in the skin immune system has already been referred to, and it is interesting to note that the "chickenwire-like" appearance (Gawkrodger et al., 1987) of HLA-DR expression along cellular membranes in focal areas of keratinocytes, closely resembles the appearance of the surface antigen described by Pass and Maizel (1973) in wart tissue. Further studies on this are warranted.

Table 6 Activity and depletion of local immune cells in HPV-associated lesions

Lesion	No of lesions studied	Local Immune Cell Involvement LC	T cells	$T_{h/i}/T_{s/c}$	NK	Reference
Verruca vulgaris	2	↓	↑	↑	.	Gatter et al., 1984 b
Regressing plane warts	?	<-->	↑	↑	↑	Iwatsuki et al., 1986
Non-regressing cutaneous warts	76	↓	↑	<--> or ↓	.	Chardonnet et al., 1986
Regressing plane warts	8	↑	↑	.	.	Oguchi et al., 1983 a,b
WVI of cervix	4	↓	↓	↓	.	Morris et al., 1983
CIN	3	↑	↑	↓	.	
Normal adjacent skin	5	<-->				
Progressing cervical lesions (CIN + HPV)	?	↓	↑	↓	.	Syrjanen et al., 1984
WVI		↓	.	.	.	McArdle & Muller., 1986
CIN	79	↑	.	.	.	
WVI + CIN		<-->				
CIN + HPV	19	↓	.	.	.	Tay et al 1987., a)
CIN + HPV	15	.	↓	↓	.	" b)
CIN ± HPV	18	.	.	.	↑(78%)	" c)
HPV in cervix		↓				Hughes et al., 1988
HPV + CIN		↓				
CIN I & II	67	↓				
CIN III	1	↓				
Controls		<-->				
CIN		↑				Hawthorn et al., 1988
CIN + HPV-18		↓				

↑ = increase; ↓ = decrease; <--> = no change in proportion of cells compared with normal skin

4.4 HPV Infections in Immunosuppressed Patients

HPV infections are the second most frequent viral complications in immunosuppressed individuals after infections with herpesviruses. The high incidence of warts after more than a year of immunosuppression in renal allograft recipients (RAR) was first noticed by Spencer and Andersen (1970) with 42% of such patients showing cutaneous warts, and in Lothian this figure has increased to 87% of RAR after 5 years of immunosuppression (Rudlinger et al., 1986). Koranda et al. (1974) also noticed a similarly high incidence of warts in RAR which began appearing about 8 months after transplantation. Like Spencer and Andersen, these workers felt this represented a reactivation of latent virus rather than a primary infection. All of the warts appeared on sun-exposed areas, mainly on hands, but also on face, arms, legs and feet, and generally were of the plane wart type.

Multiple flat warts in an RAR were shown by Pfister et al. (1979) to be caused by HPV-3, but in 1980, Lutzner and coworkers reported HPV-5 induced skin lesions in two such patients. In one of these, the lesions were of a warty type on the back of the fingers, but in the other patient HPV-5 was found in insignificant multiple pityriasis versicolor-like lesions of the arms and trunk and also in an ulcerated Bowenoid carcinoma in situ on his thumb. This was the first demonstration of the potentially oncogenic HPV-5 in any person other than EV patients, although Koranda et al. (1974) had one RAR with similar EV-like wart lesions which transformed with time into multiple squamous cell carcinomas. Of course, the HPV-type could not be determined at that time. Since then, HPV-5 DNA has been reported in plaque lesions from an RAR in Edinburgh (Rudlinger et al., 1986) and in "verrucous keratosis" lesions from three RAR in Minneapolis (Van der Leest et al., 1987). Very recently, a further three Edinburgh RAR with multiple squamous cell carcinomas have been found to harbour HPV-5 in their lesions (Barr et al., 1989). Nevertheless,

the predominant type of wart seen in the Edinburgh study was the common wart and likewise in the Minneapolis study, HPV-2 was the virus type detected most frequently. Indeed, in the single malignant cutaneous neoplasm in this group, HPV-2 was found (Van der Leest et al., 1987), and recently in basal cell carcinomas from two immunosuppressed patients (one RAR, one with systemic lupus erythematosis (SLE)), HPV-2 was also reported (Obalek et al., 1988).

Renal transplant patients suffer from an increased incidence of malignant tumours, particularly lymphomas and skin cancers (Kinlen et al., 1979). Koranda et al., (1974) noted that the skin cancers increased in incidence with time after transplantation, and were confined to areas of skin exposed to UV light. The duration of sunlight exposure further increased the incidence, just as it appeared to do in EV patients (Lutzner et al., 1983).

The increased frequency of epidermal malignancies and viral infections in RAR result from compromised cutaneous immunosurveillance, in turn attributable to immuno-suppressive therapy (Marshall, 1974; Penn, 1975). There is experimental evidence to suggest that the APC capacity of LCs in RAR is reduced by azothioprine and steroids as shown by the failure of some patients to become sensitised to DNCB (ten Berge et al., 1981).

In addition, the effectiveness of UV light in depleting LC of their surface markers including Class II antigens (Aberer et al., 1981) and the reduced LC density, disordered distribution and altered morphology observed on sun-exposed flexor surfaces in RAR led Sontheimer et al. (1984) to suggest that the combined effects of UV plus the mutagenic effects of azathioprine might lead to tolerance and tumour development. In recent years, azathioprine treatment has been replaced by cyclosporin which itself increased the risk of neoplasms, particularly non-Hodgkin's lymphoma and basal and squamous cell carcinomas (Brown et al., 1988). These workers noticed a reduction of CD1 expression on LC in a patient with psoriasis treated with

cyclosporin. Gabel et al. (1987) found decreased epidermal LC right from day 1 of treatment with cyclosporin and steroids in RAR, with a distortion of their distribution and a loss of dendrites, but were unable to distinguish between a decrease in HLA-DR expression and a decrease in number of cells. LC in HPV lesions in RAR remains to be investigated more thoroughly.

A marked decrease in LC numbers has been recorded within the lesions of EV patients (Haftek et al., 1987), all of whom showed reduced CMI in terms of anergy to DNCB sensitisation, reduced mitogenic response and reduced T cell counts. However, no significant difference in LC density in normal skin from EV patients compared with controls was observed suggesting that in this disease, the initial defect is not in the LCs but more probably in some aspect of T cell function, which in turn would explain why inflammatory infiltrates are never seen in EV lesions (Haftek et al., 1987).

In 1975, Morison noted an increased frequency of warts in patients with defects in CMI other than those who had been immunosuppressed for transplant purposes (Morison, 1975b). In particular, people with Hodgkins disease, malignant lymphoma and chronic lymphocytic leukaemia appeared to be at risk. Patients with genetic defects of CMI also have an increased susceptibility to development of multiple treatment-resistant warts (Lutzner, 1982) and of course the best studied of these groups are the EV patients.

A vast increase in anogenital lesions associated with HPV has been detected recently in groups of immunosuppressed individuals. Forty-three per cent of renal allograft recipients in a recent study were found to have warts mainly in the anogenital region (Penn, 1986) and indeed, it has been said that immunodeficient patients are an in vivo laboratory in which to study the natural history of HPV and its oncogenic effects on the anogenital tract (Sillman and Sedlis, 1987). The risk of CIN in renal transplant patients is considered to be between 8-16 times that of the normal population (Sillman and Sedlis, 1987) and

in a recent local study, 53% of such patients had cervical abnormalities (Alloub et al., 1989). Although the HPV-DNA detection rate was similar for both transplant and control patients, HPV-16/18 was detected more than four times more frequently in the transplant group. In female patients with Hodgkin's disease, 46% had cervical abnormalities, many of which were HPV associated (Katz et al., 1987) and in HIV patients, extensive anogenital warts are common (Kent et al., 1987; Forman and Prendiville, 1988). In this latter group, a significant reduction in the Class II expression of skin LC has been observed (Belsito et al., 1984), either as a consequence of HIV infection or of the opportunistic infections such as HPV so commonly associated with HIV. It seems likely that the importance of HPV associated diseases in patients with AIDS will increase greatly as the number of patients and their length of survival increases.

5. Aims of this Study and Approaches Employed

From the reports described above, it can be seen that much knowledge has been gained in recent years concerning the molecular biology of papilloma viruses and their associations with clinical lesions. Until recently, the host response to the infective agent was a neglected area, and this study was designed to examine the immune responses to HPVs in patients with dysplastic abnormalities of the uterine cervix.

This thesis reports an analysis of cellular immunity assessed by studies of peripheral blood lymphocyte responsiveness to HPV proteins and to non-specific mitogens and an analysis of humoral immunity by indirect ELISA, in an attempt to establish the role played by HPV in CIN. Virus structural proteins derived from HPV-1, HPV-2 and BPV were readily produced from wart tissue, and fusion proteins of specific HPV types were kind gifts. Attempts were made to supplement the sources of genital wart antigens by subcutaneous or sub-renal capsule implantation of infected tissue in nude mice. These *in vitro* assays were complemented by a project being undertaken by Dr. R. Hughes in the same laboratory, on epidermal Langerhans cells examined immunocytochemically in similar patients (Hughes, 1988; Hughes *et al.*, 1988). In the hope that the interactions of epidermal and systemic antigen-presenting cells, infected keratinocytes and specific lymphocytes could be clarified further with appropriate antibodies, *in situ* hybridisation methods for the detection of HPV using non-radioactively labelled cloned and synthetic probes were developed.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. STARTING MATERIALS AND THEIR PREPARATION

1.1 Viruses

1.11 Bovine Papilloma Virus

BPV-1 from a cheek fibropapilloma (obtained from the Edinburgh abattoir and stored at -70℃ until use) was purified by a modification of the method described by Gissmann and zur Hausen (1976). The tissue was chopped finely and ground in a mortar with sterile sand in phosphate buffered saline (PBS) containing 200 iu/ml penicillin and 400 µg/ml streptomycin. Grinding was continued until only small amounts of macerated tissue remained. Trypsinisation was avoided as this has been shown to disrupt virions (Pfister et al., 1977). The supernate was clarified by low speed centrifugation and virus pelleted by ultracentrifugation at 80,000g for one hour. The pellets were resuspended in PBS + 0.01M EDTA (PE) by repeated suction through a needle and syringe. Virus was purified by mixing thoroughly with 40%. caesium chloride in PE to give a refractive index (R.I.) of $\geqslant$1.3660. The mixture was ultracentrifuged at 110,000g for 24 hours at 18°C and a single band could be seen. Fractions containing this band (with R.I.s from 1.364 to 1.366 corresponding to densities of 1.33 to 1.35 g/ml) were pooled and subjected to a second density gradient centrifugation. The dense band with a density of 1.33-1.35 g/ml was removed and dialysed against several changes of PE overnight at 4°C. A diffuse band containing more empty particles was seen in some preparations. The protein content of the purified virus preparation was estimated by the Lowry method (Lowry, 1951), and concentrations ranging from 0.3-0.5 mg/ml were obtained in different preparations. The virus was filter-sterilised aliquoted and stored at -70°C.

1.12 Human Papilloma Virus

HPV-1 and HPV-2 induced lesions were identified clinically by the

staff of the Wart Clinics of the Department of Dermatology, Royal Infirmary of Edinburgh and particularly by Dr. M. Nolan and Dr. E.C. Benton who ensured that parings from each type of lesion were kept separately and placed in PBS with antibiotics for transportation to the laboratory. Purified virus preparations were obtained as for BPV-1 using two cycles of density gradient ultracentrifugation (Fig 8b and c). The preparations assumed to be HPV-1 and HPV-2 were typed by Southern blotting following restriction endonuclease digestion by Dr. B. Barr, and within the limitations of the system were found to be pure.

Purified HPV-1 preparations contained 0.2-0.9 mg/ml, while pelleted but unpurified virus contained 0.7mg/ml. Purified preparations of HPV-2 contained 0.2-0.4 mg/ml.

HPV-11 isolated from a condyloma acuminatum and grown in human foreskin cells following implantation under the renal capsule of the nude mouse (Kreider et al., 1986) was the kind gift of Dr. J.W. Kreider, the Milton S. Hershey Medical Centre, Hershey, Pennsylvania. As a result of delay in transit, only a few intact particles were observed in the electron microscope (Fig 8d).

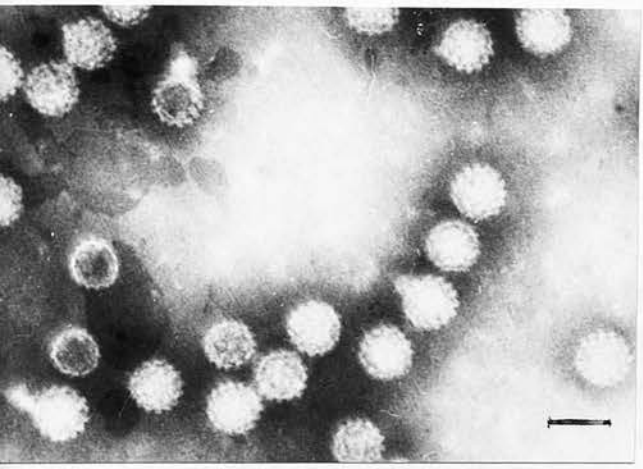
1.13 Herpes Simplex Virus

A clinical isolate of herpes simplex virus (HSV) type 1 cultured in Vero cells and plaque purified (Howie et al., 1986) was kindly provided by Mrs. J. Maingay at an infectivity of 6×10^7 pfu/ml. (plaque-forming units). It was inactivated by uv irradiation of 300 mamps/cm^2 for one hour before use.

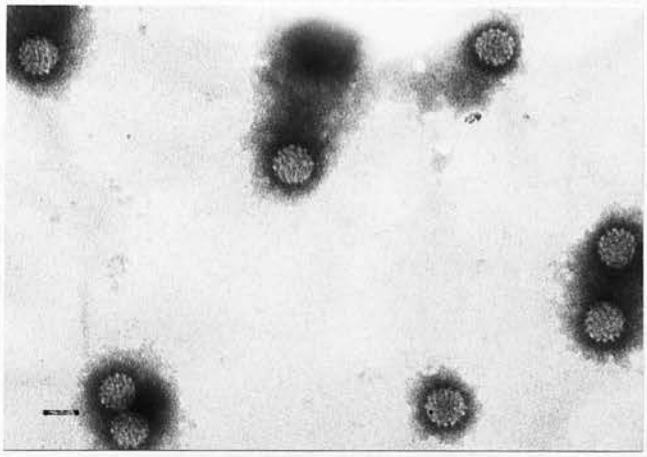
1.2 HPV Fusion Proteins

1.21 Fusion Proteins of Late and Early Genes of HPV-1 and HPV-2

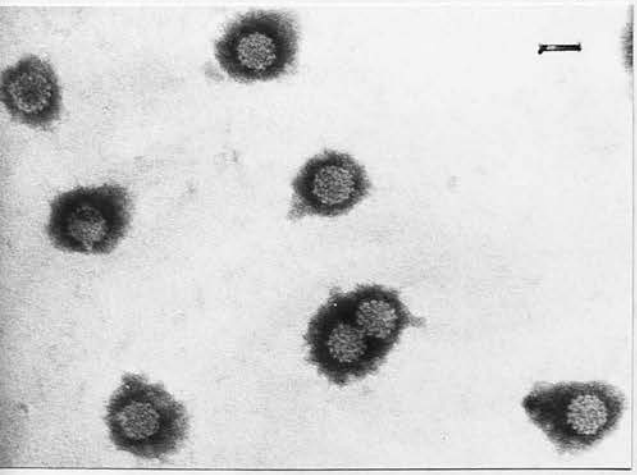
Three fusion proteins derived from the late genes of skin warts were kindly provided by Dr. J. Doorbar (Department of Cancer Studies,



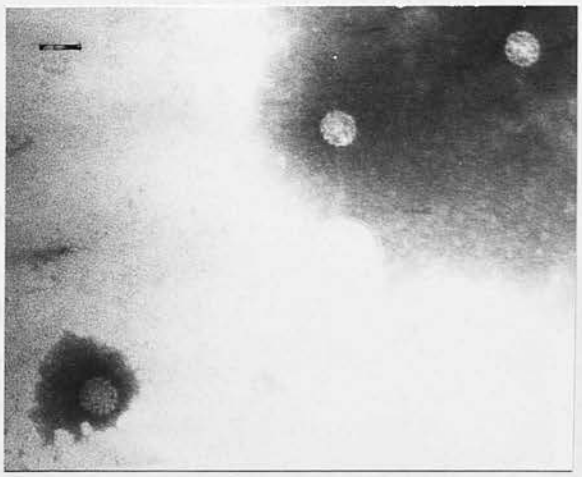
a



b



c



d

Fig 8

Extracts of HPV-1, HPV-2 and HPV-11 negatively stained with phosphotungstic acid.

(a) unpurified HPV-1
(b) purified HPV-1
(c) purified HPV-2
(d) HPV-11

Bar = 50nm

University of Birmingham). These were the C-terminus portions of HPV-1a L2 protein (nucleotides 4855-5581) and HPV-2 L2 protein, and the middle portion of the HPV-1 L1 protein (nucleotides 5581-6357). In addition HPV-1a E2 C-terminus protein was also provided. Plasmid vectors of the pEX series were used to produce recombinants with E.coli K12 as the host strain. The fusion proteins were prepared from large scale mid-log phase cultures by lysozyme lysis and boiling in sodium dodecyl sulphate (SDS) and mercaptoethanol (2-ME) followed by separation on Sepharose CL-4B (Doorbar et al., 1986). The aliquots received contained 0.15 mg/ml (HPV-1aL1), 0.3 mg/ml (HPV-1aL2) and 0.5 mg/ml (HPV-1aE2; HPV-2L2) of protein in a buffer containing PBS and residual SDS. The SDS was removed by centrifugation at 4°C and the supernates aliquoted and stored at -70°C.

1.22 Fusion Proteins of Early Genes of HPV-16 and HPV-18

Fusion proteins of the E6 ORF of HPV-16 and HPV-18 and of the E4 ORF of HPV-16 were kindly provided through Dr. L. Crawford, Imperial Cancer Research Fund, London with the co-operation of his staff, Dr. L. Banks and Dr. T. Crook. These were produced as follows :-

HPV-16 and HPV-18 E6 were synthesised in E.coli AR120 cells using pAS-1 constructs as described by Matlashewski et al. (1986) and Banks et al. (1987). The cells were extracted three times in 70% formic acid, boiled with dithiothreitol, lyophilised and separated by Superose 12 fast protein liquid chromatography. E6 control protein containing no HPV sequences was similarly prepared. HPV-16 E4 β-galactosidase fusion protein and a control protein lacking HPV sequences were produced by transcription from pUR vectors followed by lysozyme lysis and separation on Sepharose CL-4B. (Matlashewski et al., 1986). The proteins were received at a concentration of 25μg/ml and were suspended in a buffer of 0.02%SDS, 50mM TrisHCl pH7.5 and 5% glycerol. They were aliquoted on receipt and stored at -70°C.

1.3 HPV DNA

1.31 Cloned HPV DNA

HPV-1a DNA cloned into plasmid pBR322 (Burnett and Gallimore, 1985) was used with permission of Dr. P.H. Gallimore from material originally supplied by Dr. T.S. Burnett, Department of Cancer Studies, University of Birmingham to Dr. R. Rudlinger. Cloned HPV-11 (Gissmann et al., 1982b) and HPV-16 (Durst et al., 1983) also in pBR322 were kindly made available by Dr. H. zur Hausen, Heidelberg, FRG. All cloned material was provided in transfected E.coli HB101 cells stored in 40% glycerol.

The basic media for growth of the bacterial strain were LB (Luria-Bertani) broth containing 1% Bacto-tryptone, 0.5% Bacto yeast extract (both from Difco, East Molesey) and 1% NaCl, pH7.5 and LB-agar (1% agar in LB broth). Ampicillin was added at 50µg/ml to both liquid cultures and plates.

HPV-DNA was produced by amplification of the plasmid in this rich medium using methods described by Maniatis et al. (1982), and are included for completeness. To obtain large scale colonies, one loopful of stored cells was plated onto LB-agar and incubated. A single colony was placed in 10ml of LB broth, incubated and on the following day, 0.1ml of culture was inoculated into 25ml of the same broth in a conical flask and incubated at 37°C with orbital shaking for approximately 4 hours until the optical density at 600nm (OD_{600}) was 0.6. This late log phase culture was transferred to a further 500ml of broth and incubated with shaking for exactly 2½ hours when the OD_{600} was approximately 0.4. Chloramphenicol (2.5ml of 34mg/ml solution in ethanol) was added and the cultures incubated for a further 12-16 hours. The plasmid was extracted from the cells by alkaline lysis. Washed bacterial cells in 100ml of ice cold STE (100 mM NaCl + 10mM Tris/HCl pH8.0 + 1mM EDTA pH8.0) were resuspended in 10ml Solution I (50 mM glucose + 25mM Tris/HCl pH8.0 + 10mM EDTA)

containing 5mg/ml lysozyme (L-6876, Sigma Chemical Co. Ltd., Poole). After 5-10 minutes at RT, 10ml of freshly made Solution II (0.2N NaOH + 1% SDS) was added and mixed. After a further 10 minutes on ice, 7.5ml of Solution III (5M potassium acetate, pH 4.8) was added, the contents mixed sharply and again left on ice for 10 minutes. The suspension was centrifuged at 32,000g for 20 minutes at 4°C so that bacterial DNA and debris formed a tight pellet. To the supernate was added a 0.6 volume of isopropanol for 15 minutes at RT. After centrifuging at 12,000g for 30 minutes, the pellet was washed with 70% ethanol and finally dried.

The extracted DNA was purified on a caesium chloride gradient. The pellet was dissolved in 8ml TE (10mM Tris/HCl pH8.0 + 1mM EDTA). One gram of CsCl per ml was added and 0.8ml of ethidium bromide (10mg/ml stock, E8751, Sigma). The suspension was ultracentrifuged for 40 hours at 130,000g at 18°C. Two "reddish" bands were observed, the upper one due to linear bacterial DNA and linearised plasmid DNA, while the lower one contained closed circular plasmid DNA. The lower band was collected by needle from the side of the tube. Ethidium bromide was removed with 1-butanol saturated with water. The lower aqueous phase was re-extracted 4-6 times until all pink colour had gone and dialysed against several changes of TE. It was then aliquoted and stored at -70°C. The amount of DNA was estimated spectrophotometrically at 260nm and 280nm. Between 0.05-0.1mg/ml were obtained with purity ratios (OD_{260}/OD_{280}) of 1.70-1.96. Plasmid pBR322 lacking an HPV insertion was similarly amplified, extracted and purified to act as a control. The specific HPV sequences were not separated from the plasmid vector.

1.32 Synthetic HPV Oligonucleotides

Synthetic oligonucleotides were synthesised on an Oswel Gene Synthesiser in the Department of Chemistry, University of Edinburgh by Dr. Tom Brown, using B-cyanoethyl phosphoramidite chemistry. The

DNA was produced as 2μmol contained in 1ml of distilled water and had -OH 5' and 3' termini. It was purified by reverse phase high performance liquid chromatography (HPLC) and subsequently desalted by gel filtration.

Synthetic oligonucleotides of 30 bases in length with sequences from within the E6 ORF of HPV-1a, HPV-6b and HPV-16 were prepared following comparison of published sequence data (Danos et al. (1982) for HPV-1a; Schwarz et al. (1983) for HPVb6 and Seedorf et al. (1985) for HPV-16). Alignment of the sequences to allow comparison of the E6 proteins was described by Giri and Danos (1986) and it was decided to select the 30 nucleotides which occurred immediately downstream of the ATG start codon of each HPV type (Fig. 9).

The entire GenBank database (12537 nucleotide sequence files) was searched for homologies to the chosen oligonucleotide sequences using the University of Wisconsin Genetics Computer Group software (Devereux et al., 1984) by Dr. Richard Bingham, Department of Veterinary Pathology, University of Edinburgh. Variable levels of mismatching were permitted, mismatches of 9 or more bases being assumed to be insignificant. Only two comparable sequences were found. These were (i) HPV-11 differing from HPV-6b in 4 bases, (see Fig. 9) and (ii) a sequence from within the mouse macrophage Fc-γ-RB-2 cDNA (Ravetch et al., 1986) which had seven mismatches when compared with the sequence selected for HPV-16.

1.4 Specimens from Patients and Controls

1.41 Blood Samples

Ten to 20 ml of venous blood were obtained from patients attending the Lothian Area Colposcopy Clinic and collected in preservative-free heparin (H7005; Sigma; 0.1ml of 1000 units/ml of sodium salt per 10ml of blood), with the help of Mrs. Muriel Murray and the co-operation of the Colposcopy Clinic Staff. The patients were

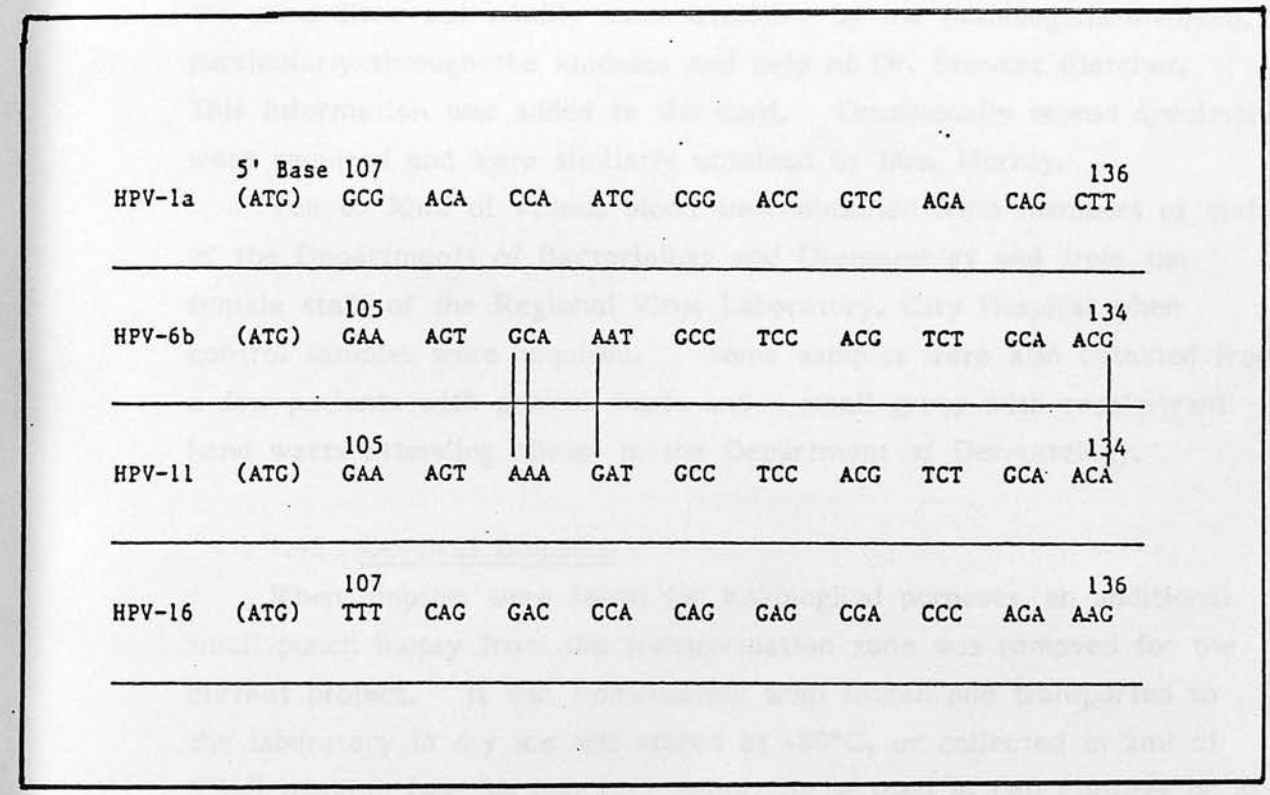


Fig 9. Oligonucleotide sequences of 30 bases in length selected for HPV-1a, HPV-6b and HPV-16, and including HPV-11 for comparison.

asked several questions by Mrs. Murray including the occurrence of past or present warts and their site, smoking history and contraceptive use, from which she compiled a card index of patient details (Fig 10). Information from the histological report of cervical biopsies obtained at the same time was readily made available by the pathologists involved, particularly through the kindness and help of Dr. Stewart Fletcher. This information was added to the card. Occasionally repeat specimens were required and were similarly obtained by Mrs. Murray.

Ten to 30ml of venous blood were obtained from members of staff of the Departments of Bacteriology and Dermatology and from the female staff of the Regional Virus Laboratory, City Hospital when control samples were required. Some samples were also obtained from a few patients with genital warts and a small group with recalcitrant hand warts attending clinics in the Department of Dermatology.

1.42 Cervical Biopsies

When biopsies were taken for histological purposes, an additional small punch biopsy from the transformation zone was removed for the current project. It was immediately snap frozen and transported to the laboratory in dry ice and stored at -80°C, or collected in 2ml of RPMI medium (see Section 2.12 below) to be used in cell cultures or in transplantation into nude mice.

1.43 Sections of Paraffin-embedded Cervical Biopsies

For in-situ hybridisation studies, sections of formalinfixed paraffin embedded cervical tissue were provided by the Department of Pathology with the permission of Professor C.C. Bird and the help of Dr. Fletcher, Dr. Kathryn McLaren and Mr. Kenny Rae and his staff. From requested biopsies, standard sections were cut and floated onto slides coated with 1% poly-L-lysine or chemically silanated (vide infra).

Pat No.:
Name...: [redacted]
DcB....: 22/06/62
Cons...: GES

Visit No. 2 FU
Date 3/3/88
VRL No. 88072

Past medical history

Yes Warts @ 12 yrs T.Z. 3/4/87 ① < 25%
Possibly nodule finger & hand at present
verruca plantaris at 9 yrs

Smoking in past 12 months 3 per day
still ups

No. of pregnancies

C +O

D.O.D.

L.M.P. 19/2/88

Current contraception

8 None
Previous condom
20/4

Histology

3/4/87 1 o.c sq - columnar. metaplasia dysplasia is probably absent 1(K-).
[clinic assessment " found definite area of epithelial abnormality around external os"]

Treatment

3/9/87 Cold coagulated

Disposal

Routine review appt.
21/9/88

Specimen

Blood - 20 ml
(H)

Fig. 10 Card layout used for compilation of patient details obtained at the time of specimen collection and from pathological reports.

1.44 Cervical Secretions

Cervical secretions were obtained from a small number of patients attending the Colposcopy Clinic with the help of Dr. Rhona Hughes and Dr. Sheila Bain. Glass bottles containing 2ml sterile distilled water were provided. This was introduced into a 5ml syringe to which was attached a needle and a 10cm length of polyethylene tubing (19-0040-01, Pharmacia Ltd.) sterilised by ethylene oxide. The water was flushed into the cervix and the secretions aspirated into the syringe and then expelled into the original bijou. Secretions were transported to the laboratory as quickly as possible, aliquoted and stored at -20°C.

1.45 Rabbit anti-HPV Serum

A preparation of purified HPV from pooled hand warts and shown by EM to be rich in virus particles was disrupted with 1% SDS + 0.5% 2-ME, boiled for one minute and emulsified in Freund's incomplete adjuvant. It was inoculated subcutaneously into two sites on the scruff of a female New Zealand white rabbit. Four injections at fortnightly intervals with 1ml of the preparation were given. The rabbit was bled from the ear two weeks later and found to have HPV antibodies. Four years later a booster dose of purified HPV-2 similarly disrupted, was administered in Freund's incomplete adjuvant as before and the animal was bled out two weeks later.

1.5 Mice

Homozygous nu/nu mice (nude mice) were obtained by the mating of nude males with heterozygous females of Balb/c background. A small colony was maintained at the Regional Virus Laboratory, City Hospital, Edinburgh. The mice were housed in a separate room at a constant temperature of 22°C, but were not otherwise barrier maintained. They were fed on autoclaved small animal pellets (Special

Diet Services Ltd., Witham) and given sterile drinking water changed two or three times per week. More recently, 0.01mg/ml trimethoprin and 0.05mg/ml sulphamethoxazole were added to the drinking water (Kreider et al., 1986). Experimental mice were always caged with at least one other mouse, either another experimental nude sibling or a haired sibling. The mice survived well, regularly living until 6-8 months of age when they succumbed to the wasting diseases frequently observed in nude mice. Occasionally, eye infections developed in individual animals, but no other microbiological problems were encountered.

1.51 Human Foreskin Tissue for Implantation

Human foreskin from children was obtained from Mr. William Bissett, Royal Hospital for Sick Children, Edinburgh. It was wrapped in moistened sofratulle gauze and transported to the laboratory in a sterile container. The foreskin was cut open, dermal tissue was scraped away with a sterile scalpel and the remaining skin cut into pieces approximately 2 x 1mm in size. Foreskins were used for implantation in nude mice within 3 days.

2. METHODS

2.1 In vitro Assays of Lymphocyte Recognition and Activation

2.11 Antigen Preparation for LPA

Purified antigens of BPV, HPV-1 and HPV-2 produced as described in Section 1.1 were treated further to reveal internal antigens. Detergent disrupted PV were prepared by mixing an equal volume of virus with freshly prepared 2% sodium dodecyl sulphate (SDS) and 2% 2-mercaptoethanol (ME), boiling for 1 minute followed by ultrasonicating for 1 minute and diluting in RPMI. (SDS.ME PV).

Glycine extracts of BPV and HPV-2 were prepared by disruption of purified virions in freshly prepared 0.1M glycine buffer, pH9.0 (Booth et al., 1979) followed by three cycles of freezing and thawing. The suspension was bench centrifuged and filter sterilised before use. The extract of BPV-1 was found to have a protein content of 0.4mg/ml and that of HPV-2 was 0.9mg/ml. Glycine extracts of bovine skin and human keratoses were similarly prepared.

Glycine extracts of cervical biopsy tissue were prepared to investigate the possibility of a relevant cell-associated antigen. The tissue was minced with scalpel blades, and the cells dispersed in collagenase-dispase solution (Boehringer-Mannheim Ltd.) for 1½-2 hours at 37°C. The cells were pelleted, washed with RPMI and resuspended in 0.5ml glycine buffer as above. After freezing and thawing 3 times, sonication for 1 minute and clarification by light centrifugation, the supernate was filter sterilised. Where possible, the protein content of the extract was measured and concentrations of 0.6-3.7mg/ml were obtained.

2.12 Standard Lymphocyte Proliferation Assay

Peripheral blood mononuclear (PBM) cells were separated from red cells on Lymphopaque gradients (Nyegaard, Oslo). They were washed 3

times with sterile PBS containing 0.2% heparin and resuspended in 5ml RPMI containing 15% autologous plasma (complete RPMI; made from RPMI 1640 (Northumbria Biologicals Ltd., Cramlington) containing 2mM L- glutamine, 5 x 10^{-5}M 2-mercaptoethanol (2-ME), 100 iu/ml penicillin, 200 µg/ml streptomycin, 100 µg/ml gentamycin and 2µg/ml fungizone). On a few occasions 15% foetal calf serum (FCS) was added in place of autologous plasma. A 1in10 dilution of cells was counted (0.1ml cells + 0.5ml red cell lysis buffer (made with 10ml 0.17M Tris/HCl pH7.2 and 90 ml 0.16M ammonium chloride) + 0.4ml 0.1% trypan blue in PBS) and the cells finally resuspended at a concentration of 1 x 10^{6}/ml.

Wells of sterile flat-bottomed microtitre plates (Falcon 3072; Becton Dickinson U.K. Ltd., Cowley) were inoculated with 200µl of cell suspension and 2-5µl of mitogen or antigen was added.

In general, quintuplicate wells were set up for each antigen. The mitogens used were concanavalin A (Con A; C2631; Sigma), phytohaemagglutimin (PHA; Wellcome Reagents Ltd., Beckenham) and pokeweed mitogen (PWM; Gibco Ltd., Paisley). The antigens included purified BPV, HPV-1 and HPV-2; glycine extracted BPV and HPV-2; unpurified HPV-1; SDS.ME BPV-1, HPV-1 and HPV-2; glycine extracts of bovine skin, human keratoses and cervical cells; fusion proteins as described in Section 1.2 and uv-inactivated HSV. The antigens were added at concentrations varying from 0.04-25µg/ml representing 10-fold dilutions of stock preparations from 10^{-1} to 10^{-4}.

The plates were covered with Transpaseal ^{TM} self-adhesive plastic, a small hole was punctured over each well with a sterile needle and the plates were incubated in a humidified atmosphere of 5%CO_2 at 37°C for varying times, although 7 and 8 days were most frequently used. For the final 18-24 hours, 0.75µCi ^{3}H-methyl thymidine (Amersham International, Little Chalfont) was added (10µl of label in PBS) to each well. The cells were harvested using an Ilacon II or a Dynatech Automash 2000 cell harvester onto Titertek or Whatman filter discs.

After drying, the discs were placed in toluene based scintillation fluid and counted for 1 minute in a Packard Automatic scintillation counter.

Lymphocyte proliferation was expressed as the stimulation index (S.I.) calculated by dividing the mean count in the presence of antigen by the mean count in the absence of any antigen. Where 5 or more wells per antigen dilution were set up, the highest and lowest counts were excluded and the mean, standard deviation (S.D.) and standard error (S.E.) for each group calculated. Where there was wide variation between wells, or where there were fewer than 5 replicate wells, none were excluded from the analysis.

2.13 Addition of Lymphokines

In order to stimulate any activated lymphocytes, interleukin-2 (IL-2; Boehringer-Mannheim Ltd.) was added at concentrations ranging from 0.1% to 20% to both antigen-containing and control wells, either on day 0 or after activation may have commenced on days 2,3 or 4. Very occasionally, IL-1 produced in the Department of Pathology by Dr. A.E. Dewar, was added at a dilution of 1 in 40 at the beginning of the culture period to both antigen and control wells.

Supernate from PBL cultured with antigen was added to fresh PBM in the presence of PV antigens to see if this was suppressive.

2.14 Alteration of Antigen-presenting Cells

Cervical biopsies from 10 patients were chopped and dispersed in collagenase-dispase as above, then pelleted, resuspended in 1ml complete RPMI, washed once and counted. They were added to PBM generally at a concentration of 4% to control wells without antigen and to test wells containing various papilloma antigens. The cultures were harvested after 8 days.

On a single occasion, the washed cells were purified on a Percoll (Nyegaard, Oslo) gradient of 70%, 55% and 20% in RPMI. The gradient was spun for 20 minutes at 400g and the visible band at the

55/20 junction was removed, washed twice with PBS, resuspended in RPMI, counted and added to cultures at 4%.

On a few occasions, adherent cells were removed from PBM by placing 8 x 10^6 cells in complete medium in an etched plastic petri dish (Costar 3160; NBL). At 15, 30 and 60 minutes thereafter, the non-adherent cells were pipetted off, the adherent cells scraped off with a plastic scraper (Costar C3010; NBL) and added back to the non-adherent cells at varying percentages from 0.3%-10%.

T-lymphocytes were purified from the non-adherent cells by passage over nylon wool columns (Julius et al., 1973). Adherent cells were scraped off the glass and added to the T cell population in concentrations ranging from 0.3-5%. The LPA was then set up in the standard way but included wells which contained T cells and adherent cells, T cells alone and adherent cells alone, each with and without antigen.

2.15 Removal of $CD8^+$ Cells by Panning

Two ml of PBM containing 7 x 16^6 cells/ml were spread on a plastic petri dish which had been coated with 1:10 anti-$CD8^+$ monoclonal antibody (Dako T8) in filter-sterilised carbonate coating buffer pH9.6 (as used in ELISA tests, see section 2.21) overnight at 4°C. The cells were left in contact for 2 hours at 4°C when the non-adherent cells were removed by pipette. The adherent cells were scraped off the plastic, washed gently several times, resuspended in complete RPMI and added in the ratio 3 x 10^5 to 1 x 10^6 to the non-$CD8^+$ cells. Untreated PBM, $CD8^+$-depleted PBM, and reconstituted PBM were then used in a standard LPA.

2.16 Subset Analysis before and after LPA

Fluorescent flow cytometry was used to analyse the cell subsets involved in proliferative events following PV antigen exposure. The flow cytometer used was an EPICS C (Coulter) equipped with an argon

ion laser using 200mW power and emitting light at 488nm. Five thousand to 10,000 stained cells were analysed at a flow rate of 400-500 cells/s.

On day 0, surplus cells after the LPA had been set up were divided into aliquots of 5×10^5 cells. The cells were washed in PBS + 1% normal rabbit serum (NRS) pelleted at 150g for 10 minutes and stained as described by Neill and Miller (1987) with monoclonal antibodies to label all T cells (UCHT-1, Unipath Bedford; OKT3, Becton and Dickinson Ltd.), $CD4^+$ and $CD8^+$ (Dako T4 and T8) or NK cells (Leu 7, Becton and Dickinson Ltd.). Ten microlitres of monoclonal used neat (UCHT-1 and T3) or diluted 1in2 in PBS + 1% NRS (T4, T8, leu 7) were added to the cell pellets, vortexed and held on ice at 4°C for 30 minutes. Cells were washed twice and 25 µl of a 1in40 dilution of FITC-conjugated sheep antimouse IgG $F(ab')^2$ serum was added again for 30 minutes on ice. Cells were again washed twice and finally resuspended in 1ml of PBS + 1% NRS to which was added 100 µl of neutral formalin in PBS. For B-cell staining a one-step direct fluorescent method was employed, using an FITC-conjugated rabbit antihuman IgM antiserum. An incubation time of 30 minutes at 4°C was again used.

For analysis at the end of proliferation the cultures were set up in an identical manner to the LPA except that round bottomed plates (Cel-Cult; Sterilin Ltd; Feltham) were used to facilitate pelleting. At the end of incubation, the plates were spun on a Sorvall plate centrifuge at 4°C for 5 minutes at 1000 rpm. The supernatant fluid was either discarded or collected for adding to subsequent LPA, the cells were resuspended, washed with PBS + 1% NRS and 8 µl of the appropriate monoclonal or directly labelled antihuman IgM antiserum added. The cells were again mixed and held in the dark at 4°C on ice. The staining was continued as described for cells in tubes using 10 µl of conjugated anti-mouse serum and finally resuspending in 200 µl PBBS + 1% NRS plus 20 µl 10% neutral formalin in PBS. The plates were covered with Transpaseal until analysed.

2.17 Attempts to Establish Lymphocyte Cell Lines

Attempts were made to establish cell lines of lymphocytes activated by PV antigens using the method described by Lanzavecchia et al. (1982). Two million PBMs in 10ml of complete RPMI were placed in a small Falcon flask with 500µl of 10^{-2} glycine-extracted HPV-2. After 7 days, the cells were pelleted, resuspended in 200µl of 100% Percoll onto which was layered a discontinuous Percoll gradient (60%, 50%, 40%, 20% in RPMI) according to the method of Kurnick et al. (1979). The gradient was spun at 3000 rpm for 10 minutes and lymphoblasts recovered as the visible band from the 40-50% interface. The cells were washed twice with large volumes of PBS and resuspended in complete RPMI at a concentration of $2x10^5$/ml. Three per cent IL-2 was added at this stage and again when the cells were expanded by the addition of more medium. After 3-4 weeks in culture, some cells were restimulated with antigen by mixing $5x10^5$ blasts with $5x10^6$ autologous irradiated PBM (10^7 cells in 1ml PBS were irradiated with 2600 rads for 1 minute from a Caesium 137 gamma source of 3KiC in the Department of Zoology, University of Edinburgh). After adding 250µl of 10^{-2} glycine extracted HPV-2, the cells were dispensed in 8 wells of a 24 well plate (Costar, NBL) and IL-2 was added. After 7 days the blasts were again separated on a Percoll gradient.

2.2 Assays of Antibody Production

2.21 Enzyme Linked Immunosorbent Assay (ELISA) to Detect Serum IgG to HPV

The assay developed was a modification of the micromethod originally described by Voller and Bidwell (1976) and applied to the detection of BPV viruses (El Shazly et al., 1985). Purified PV virions (HPV-1, HPV-2 or BPV) disrupted with SDS.ME (see Section 2.11) were diluted in carbonate coating buffer pH 9.6 to a concentration of 1-2 µg/ml (made up with 1.5g Na_2CO_3 $10H_20$, 2.93g $NaHCO_3$ and 0.2g NaN_3 per litre). One hundred µls were dispensed into alternate wells

of flat-bottomed microtitre plates (ELISA grade; Nunc Immuno-Plate I, 96F) and buffer containing 1% bovine serum albumin (BSA; Sigma) was added to the remaining wells as a background control. The plates were sealed and after incubating at 4°C overnight they were washed four times with washing buffer (PBST i.e., PBS + 0.05% Tween 20 + 0.1% BSA + 0.02% NaN_3) and then incubated with 100 µl per well of PBST containing 3% BSA at RT for 1 hour to block remaining combining sites on the plastic (Forghani and Schmidt, 1979). Washed plates were dried and were generally used the same or following day, but could be kept for up to 10 days at 4°C.

The plasma specimens to be tested were diluted 1 in 50 in PBST + 1% BSA and 100µl of each added to antigen coated and control wells, and incubated for $2\frac{1}{2}$ hours at 37°. After four washes, 100ul alkaline phosphatase conjugated anti-human IgG (1:500; A-3150; Sigma) diluted in PBST + 1% BSA was added for 2 hours at 37°C. The plates were again washed, and 100µl of substrate, para-nitrophenyl phosphate (pnpp; N9389; Sigma) prepared as 1mg/ml in fresh diethanolamine buffer pH9.8 without $MgCl_2$ (i.e., 80ml distilled water plus 10ml diethanolamine warmed to 37°C and the pH adjusted with N HCl). The reaction was allowed to develop for 20-30 minutes at RT before being stopped with 100µl of 3M NaOH. The absorbance of each sample was read spectrophotometrically at a wavelength of 410nm on a Dynatech platereader.

A positive and at least triplicate negative controls were included on each plate. The positive control was from a healthy member of staff who consistently gave strong reactions to HPV-1 and HPV-2 antigens during the development of the test and the negatives were selected from colposcopy patients with no known history of warts, no koilocytes or warty atypia in histological sections, and who repeatedly gave very low absorbancies during the development of the test.

The corrected absorbance for each test sample was calculated by subtracting the absorbance of the background control well from that of

the antigen-containing well. The ELISA Index (E.I.) was calculated by dividing the absorbancy of the test sample by the mean absorbance of the negative samples plus 3 standard deviations.

2.22 ELISA to detect Secretory IgA in Cervical Lavage Specimens

An indirect ELISA developed to detect secretory IgA in tears in ocular herpetic infection (Fox et al., 1986b) was modified to investigate the presence of locally produced secretory IgA in the cervix. Microtitre plates were coated with SDS.ME HPV-2 at a concentration of ug/ml as described in Section 2.21. Cervical secretions, collected as described in Section 1.24 and stored at -20°C, were doubly diluted in PBS + 5% NRS from 1 in 2 upwards, added to appropriate wells and incubated for 3 hours at 37°C. After washing, 100 µl of a 1 in 100 dilution of rabbit serum against human secretory component and conjugated with horseradish peroxidase (HRP; P166, Dako) was added and incubated for 45 mins at 37°C. Ortho-phenylene-diamine substrate (P6912; Sigma) dissolved at a concentration of 0.4mg/ml in freshly prepared citrate phosphate buffer pH6.0 (5.6g citric acid + 22.5g Na_2HPO_4 per litre) was added after washing the plates for approximately 20 minutes and the reaction stopped with 100µl 4M H_2SO_4. The absorbancies were read spectrophotometrically at 490nm.

Because baseline values of positivity were difficult to establish, secretions were considered to be reactive when they gave absorbancies 0.2. As negative controls, a 1 in 100 dilution of plasma from the same patients was included wherever possible.

2.3 Histochemical Studies

2.31 Preparation of Slides and Sections

Since most of the histochemical investigations involved in situ hybridisation (ISH) with HPV-DNA probes, it was essential to have good

tissue adhesion to slides. This was achieved at first by the coating of the slides with 1% poly-L-lysine (P7886; Sigma). Two or three drops of poly-L-lysine solution were placed on cleaned glass slides and two slides pulled across each other at right angles to spread the drops. The slides were allowed to dry by draining. Following the publication of an effective method of tissue adhesion achieved by chemical silanation of slides (Tourtellotte et al., 1987), most slides for ISH were prepared in this way. The slides were first washed for 30 minutes in 1M HCl, washed in distilled water and dipped in 95% ethanol for 30 minutes. After wiping dry, they were soaked for 3 hours in Denhardt's solution (0.02% each of Ficoll (F2637; Sigma), polyvinyl pyrrolidone (P-5288; Sigma) and BSA fraction V in water), dipped briefly in water, transferred to a 3 in 1 mixture of ethanol:acetic acid for 20 minutes and air dried. The slides were then dipped in 0.1M triethanolamine at pH8.0, acetic anhydride was added to a concentration of 0.25% and mixed vigorously for 10 minutes. After two washes in distilled water and one in 95% ethanol, the slides were again air dried, then left overnight in 1% γ-aminopropyltriethoxysilane (A3648 ; Sigma) pH3.45 at 70°. After extensive washing in distilled water the slides were baked at 100° overnight. To activate the slides, they were held in 10% glutaraldehyde in PBS for 30 minutes, rinsed briefly in water and stabilised by treating with 0.1M sodium metaperiodate for 15 minutes at RT and rinsed in PBS. Activated slide could be stored for up to 8 weeks at RT.

A much quicker method of silanation was described by Burns et al. (1987) and by Maddox and Jenkins, (1987). This involved soaking clean slides in acetone or methanol followed by immersion in 2% γ-aminopropyltriethoxysilane solution (Tespa; A3648, Sigma) in acetone for 5 minutes at RT. After a wash in distilled water the slides were left to dry overnight and could be stored on the bench for several weeks.

Most histochemical studies were carried out on paraffin sections. These were conventionally cut 5μ sections and mounted on treated

slides. They were generally held at 56°C for at least one day thereafter and stored at RT until used. Occasional cryostat sections were cut from snap-frozen biopsy tissue. Semi-serial sections, 8µ thick were cut on a Slee cryostat in the Department of Dermatology and mounted on treated slides. Some were stored at 20°C without fixation, wrapped carefully in Saran-wrap ™, while others were fixed for 10 minutes in acetone and air dried before storing at 20°C.

2.32 Labelling of HPV Probes for Hybridisation

2.321 Labelling of cloned probes

The labelling of SV-40 circular DNA was originally described using DNA polymerase I by Rigby et al. (1977). In the present study, a modification of this well established method of "nick translation" using a combination of DNA polymerase I and DNASE I was carried out using the BRL Nick Translation kit (8160SB, BRL Ltd) according to manufacturer's instructions, using a biotinylated nucleotide with an 11 atom spacer arm linking biotin to the C-5 position of the pyrimidine ring (biotin-11-dUTP; BRL Ltd). Briefly, unlabelled nucleotides were mixed with approximately 1 µg of cloned DNA of HPV-1a, HPV-11, HPV-16 or pBR322, biotin-11-dUTP and sterile distilled water in recommended proportions in a 1.5ml Eppendorf tube held in ice. A 1:10 volume of DNA polymerase I/DNAse I was added and mixed, spun briefly and incubated at 15℃ in a water bath for 90 minutes. The reaction was stopped with 300 mM EDTA pH 8.0.

Unincorporated nucleotides were separated from the labelled DNA by exclusion chromatography through Sephadex G-50 (Pharmacia). Initially columns were prepared in 5 ml syringes plugged with glass wool. The sephadex, swollen by autoclaving and washed with TE (section 1.31) was loaded into the syringe, allowed to settle and washed again with several changes of TE. The whole DNA sample (50 µl) was loaded onto the top of the gel together with 100 µl of TE washings from the Eppendorf tube. A flow rate through the column of

0.5ml/min was established and approximately 24 fractions of 3-5 drops were collected. Later on, pre-packed disposable Nick ^{TM} columns (Pharmacia) were used (see Section 2.322 below).

Two microlitre aliquots of each fraction were spotted onto nitrocellulose filter (Schleicher and Schull, Dassel, W. Germany) allowed to air dry and baked in a vacuum oven at 80°C for 30 minutes. The filter was stained using the BRL DNA detection system (8239 SA, BRL) according to manufacturer's instructions. Briefly, the filter was rehydrated for 10 minutes in Buffer 2 (3% BSA in Buffer 1 (0.1M Tris/HCl pH7.5 containing 0.1M NaCl, 2mM MgCl2 + 0.05% Triton-X-100)). The biotinylated probe was amplified using streptavidin (2µg/ml in Buffer 1; 3 ml per 100cm^2 filter) for 10 minutes at RT followed by three 5 minute washes in large volumes of Buffer 1 and detected with biotinylated alkaline phosphatase (1µg/ml in Buffer 1) for 10 minutes at RT. After two washes in Buffer 1 and two washes in Buffer 3 (0.1M Tris/HCl pH 9.5 + 0.1M NaCl + 50 mM $MgCl_2$), the filter was placed in a polythene bag and freshly made substrate was added (nitroblue tetrazolium (NBT), 75 mg/ml in 70% dimethylformamide and 5-bromo-4-chloro-3 indolyl phosphate (BCIP), 50mg/ml in dimethylformamide in the proportions 33 µl NBT + 25µl BCIP in 7.5ml Buffer 3). The bag was sealed and colour development took place in the dark within 1 hour. The reaction was stopped with 20mM Tris-HCl pH7.5 + 5mM EDTA. Labelled DNA was present in an early peak while unincorporated nucleotides gave a longer weaker trail. Fractions giving the strongest signal were pooled, aliquoted and stored at -70°C.

2.322 Labelling of synthetic oligonucleotide probes

The use of the enzyme terminal transferase for labelling oligonucleotides at the 3' end has recently been suggested to be more efficient and less expensive than labelling with polynucleotide kinase at the 5' end, at least for radiolabelled probes (Lin et al., 1987) and it was decided to try a similar enzyme system to enable the linkage of

biotin-11-dUTP to the 3' end of the oligonucleotide. This method had the added advantage of being a single step procedure which renders labelled fragments resistant to exonucleases. To ensure the addition of only one nucleotide at the end of each molecule, a labelled di-deoxy nucleotide is recommended (Amersham International plc). In the absence of a biotinylated di-deoxy residue, it was realised that heterogeneous extension of 3' ends might occur (Roychoudhury et al., 1976).

Using the Amersham 3' end labelling kit (N4005; Amersham) and biotin-11-dUTP, the following reagents were mixed in a 1.5ml Eppendorf tube held in ice : 2.5µl oligonucleotide (equivalent to approximately 5nM of DNA), 12.5µl sterile distilled water, 5µl buffer (containing sodium cacodylate, cobalt chloride and dithiothreitol and provided ready for use). 25µl biotin-11-dUTP and 10µl of terminal transferase. The reaction mixture was gently aspirated several times and incubated for 2 hours at 37°C in a water bath. The enzyme mixture was loaded directly onto a disposable Nick ^{TM} column containing Sephadex G-50 DNA grade and equilibrated with 50mM Tris/HCl pH 7.5 containing 150mM NaCl, 10mM EDTA and 0.1% SDS. The fractions (100-200µl) were collected, spotted onto nitrocellulose and the labelled oligonucleotide peak detected as described above. Fractions giving the strongest signal were pooled, aliquoted and stored at -70°C.

2.33 In Situ Hybridisation (ISH)

2.331 ISH using cloned HPV probes

Paraffin sections on coated slides were dewaxed by 2 x 10 minute washes in warm xylene, rinsed in absolute alcohol for 2 x 10 minutes, rehydrated through 75%, 50% and 25% alcohol each for 10 minutes, and finally rinsed in distilled water.

Target DNA was unmasked by a series of steps following the method described initially by Brigati et al. (1983), modified by Lewis et al. (1987) and adapted as follows :-

(a) 0.02M HCl for 10 mins followed by 10 mins rinse in PBS.

(b) 0.01% Triton-X-100 in PBS for 90 secs followed by rinse in PBS.

(c) 0.2 mg/ml Proteinase K (P0390, Sigma) in 50mM Tris/HCl pH 7.4 containing 5mM EDTA for 15 mins at RT followed by 2 x 5 min washes in PBS + 0.2% glycine to stop the enzyme reaction.

(d) 20% acetic acid at 4°C for 15 secs, followed by post-fixation in freshly prepared 4% paraformaldehyde in PBS for 5 mins.

(e) 2 x 5 min washes in PBS + 0.2% glycine.

(f) Dehydration through alcohols, 10 min each, to absolute alcohol followed by air-drying.

The probe was added at a final concentration of 400ng/ml to the high stringency buffer described by Ungar et al., (1986) in their rapid ISH method for cytomegalovirus (CMV). The buffer contained 45% formamide (deionised by mixing with mixed-bed ion-exchange resin (Bio-Rad Ag 501-X8, 20-50 mesh) for 30 mins followed by double filtration through filter paper. (Maniatis et al., 1982)), 5 x SSC (where SSC = 0.15M NaCl + 0.015 sodium citrate), 25mM sodium phosphate pH 6.5, 1 x Denhardt's solution, 250 µg/ml sheared herring sperm DNA (Boehringer-Mannheim) and 10% (W/V) dextran sulphate (Pharmacia). Twenty to 50µl aliquots of probe were spotted onto sections which were then covered with a plastic coverslip cut from an autoclavable polypropylene bag. The slides were placed on a metal tray and probe and section were denatured together by floating on a water bath at 90°C for 10 minutes. The tray was transferred briefly to a 37°C water bath and the slides placed in moist boxes at 42°C for 18 or 42 hours.

After hybridisation, the coverslips were removed by gentle agitation of the slides in a bath of 2 x SSC for at least 10 minutes. This was followed by stringent post-hybridisation washes of 2 x SSC at

60°C for 10 minutes, followed by 0.2 x SSC for 10 minutes, 0.2 x SSC at 42°C for 20 minutes followed by 0.1 x SSC for 10 minutes, and a final rinse in 2 x SSC. Occasionally, a shorter series of less stringent washes (3 x 5 minutes in 2 x SSC) was used.

Hybridised DNA was detected using the BRL-DNA detection system according to manufacturer's instructions as follows :-

Slides were washed in Buffer 1 for 10 minutes at RT followed by a 20 minute wash in the same buffer containing 3% BSA (Buffer 2) to block non-specific binding. The biotinylated DNA was amplified by streptavidin and biotinylated alkaline phosphatase and detected with NBT/BCIP substrate as described above in Section 2.321. The slides were placed in a moist box in the dark for 1-2 hours. Although the manufacturers claim that maximal colour development is usually obtained within 4 hours, it was found that periods greater than 2 hours led to excessive background staining. The reaction was stopped by immersing in 20mM Tris/HCl pH 7.5 + 5mM EDTA. The slides were usually mounted in 50% glycerol in PBS pH 8.2 or in glycerol-gelatin (10g gelatin dissolved in 60ml distilled water by heating and added to 70ml glycerol, with Thiomersal (0.25%) added as a preservative) generally without counterstaining, although occasional counterstaining with 2% methyl green was used.

2.332 ISH using synthetic Oligonucleotide probes

One of the aims in developing synthetic oligonucleotide probes was to find a quicker method of ISH which was at least as sensitive. The protocol developed therefore arose from simplification, shortening and deletion of parts of the method described above for cloned probes. The most satisfactory procedure was found to consist of a pre-hybridisation protocol limited to 0.02 M HCl for 10 minutes and Proteinase K (0.2mg/ml) for 15 minutes at RT with the enzyme reaction being stopped by 0.2% glycine in Tris-buffered saline (TBS; 5mM Tris/HCl pH 7.4, 150 mM NaCl) followed by dehydration through

alcohols and airdrying. Probe was added in the proportion 1 in 10 (approximate concentration of probe in buffer was 250ng/ml) to the same high stringency buffer and denaturation was carried out as described above. Hybridisation was again at 42°C but was limited to 2 hours. Post-hybridisation washes were either under high or low stringency conditions as described and detection of bound DNA was again using the BRL-DNA detection system.

2.323 ISH using synthetic oligonucleotide probes and immunogold silver staining (IGSS)

A silver enhancement reaction to improve the sensitivity of non-radioactive methods of ISH was described by Burns et al., (1985) for the detection of the Y chromosone in intact cells, using a polyclonal goat antibiotin layer, followed by peroxidase conjugated rabbit antigoat and di-amino-benzidine (DAB) substrate, with the signal being amplified by silver precipitation. The availability of totally new reagents made possible further improvement to this approach to detection. Samples of monoclonal mouse anti-biotin (M743) and of colloidal gold coated rabbit anti-mouse immunoglobulins (G385) were kindly provided by Dako Limited. The detection of bound biotinylated oligonucleotides was achieved with the following protocol :-

After post-hybridisation washes in SSC, 25µl of mouse anti-biotin (1 in 20 in TBS + 1% BSA) was applied for 45 minutes at RT. The slides were washed for 2 x 5 minutes in TBS, and non-specific reactions were blocked with 20% NRS in TBS for 10 minutes. 25 microlitres of rabbit anti-mouse coated with colloidal gold were added at a dilution of 1 in 50 in TBS + 1% BSA for 1½ hours at RT. The slides were washed in distilled water for 10 minutes and the deposited gold particles were enlarged using Intense ™ II silver enhancement kit (Janssen marketed through ICN Biomedicals Ltd., High Wycombe). Equal volumes of enhancer and initiator solutions were mixed according to manufacturer's instructions and immediately applied to the sections. Silver

precipitation to give visible particles round deposited gold was observed to occur under the microscope within 10 minutes and the reaction was stopped by immersion in distilled water before "self-nucleation" resulted in high background staining.

2.34 HPV-Antigen Detection

HPV capsid antigen was detected on paraffin sections using an immunoperoxidase protocol developed in the Department of Pathology. Sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by incubating the slides in 3% H_2O_2 in water for 15 minutes followed by a 5-minute water wash. The sections were trypsinised using freshly made 0.1% trypsin + 0.1% $CaCl_2$ in distilled water for 15 minutes at 37°, washed in water for 10 minutes and in TBS + 2% NPS (normal pig serum) (TBSP) for 5 minutes. Blocking of non-specific binding of the swine second antibody was carried out using 20% NPS in TBS for 10 minutes. Rabbit antiserum to SDS-disrupted BPV-1 (Dako) was applied as a 1 in 20 dilution in TBSP for 1 hour at 37° or overnight at 4°C. After two 10 minute washes in TBSP, swine antiserum to rabbit immunoglobulins (Z196; Dako) at a dilution of 1 in 30 was applied for 30 minutes at RT, and after a further two 10 minute washes, anti-rabbit PAP (peroxidase-antiperosidase complexes; Z113; Dako) diluted 1 in 100 was added for 30 minutes at RT. After washing as before, reactions were detected following the application of DAB substrate (13033; BDH) made by injecting 5ml of 50mM Tris-HCl pH 7.5 into a 5mg ampoule of DAB. After thorough shaking to dissolve the DAB, the contents were added to a further 20ml of Tris buffer in a universal and 14µl of 6% H_2O_2 was added a minute before applying the substrate to the sections. Development was allowed to proceed for a maximum of 10 minutes before the reaction was stopped by washing with tap water for 5 minutes. Slides were examined in aqueous mountant without counterstaining.

2.35 Double Staining Methods

Paraffin sections of skin warts and cervical dysplasias were stained for both HPV capsid antigens and specific DNA. The antigen detection was carried out as described above (2.34) and resultant nuclei containing antigens stained brown. Slides were then prehybridised by either the long protocol with cloned DNA or the short protocol for synthetic oligonucleotides, hybridised for 18 hours or 2 hours respectively and detected with the BRL-DNA detection system, or with immunogold and silver enhancement for synthetic probes only. Generally no counterstains were applied before finally mounting the sections in glycerol-gelatin. Very occasionally 2% methyl green for 1 minute was used.

Antigen staining and ISH were attempted on cryostat sections using similar protocols. In addition, cryostat sections were stained for the presence of Langerhans cells using the method described by Carr et al. (1986) and adapted by Hughes et al. (1988): the monoclonal antibody DAKO-T6 (M721; Dako) was applied for 45 minutes at RT at a dilution of 1in 20 in TBSR (TBS + 2% NRS), following blocking of endogenous peroxidase with 0.3% H_2O_2 in methanol for 15 minutes and of non-specific binding with 20% NRS in TBS for 20 minutes.

Rabbit antimouse immunoglobulins conjugated to horseradish peroxidase (P161; Dako) at a dilution of 1in80 in TBSR were applied for 15 minutes at RT and after two 10 minute washes in TBSR, DAB substrate, made up as described in Section 2.34 was allowed to react for 3-5 minutes. The reaction was stopped by washing in tap water.

2.4 Attempts to Induce HPV Proliferation in Nude Mice

2.41 Subcutaneous Implantation of Cervical Biopsies

Cervical biopsies of varying grades of dysplasia obtained from the Colposcopy Clinic and transported to the Regional Virus Laboratory in RPMI, were implanted subcutaneously into 38 adult nude mice (25

female and 12 male; aged 37-110 days, x = 63 ± 20 days). The biopsies were cut into approximately 1mm^2 pieces and held in a drop of medium in the end of a 16-guage needle while the mice were briefly anaesthetised with ether. The skin of the dorsal flank was held with forceps, an incision was made with the needle and the biopsy injected into the subcutaneous space with a bleb of air. The inoculation sites were observed weekly during the lifetime of the animal. When the animal showed signs of wasting it was killed with ether, and any lesion excised and placed in formol saline.

2.42 Implantation of HPV-infected Foreskin under the Renal Capsule

Human foreskin from young boys was prepared as described in Section 1.51. Two or three pieces were suspended in 0.1 ml of HPV-11 suspension for 1 hour at 37° as described by Kreider et al., (1986). Adult mice were anaesthetised with Nembutal (Ceva Ltd., Watford; 0.1ml of 1in10 dilution in warm normal saline per 10g of body weight) inoculated intraperitoneally. A small paravertebral subcostal incision was made and the kidney exposed by gently squeezing the sides of the mouse. The renal capsule was nicked and a single fragment of HPV-11 exposed foreskin inoculated into the space from a pasteur pipette. The wound was closed with sutures (17mm taper-cut "Mersilene" sutures; Ethicon Ltd., Edinburgh) if necessary or simply by spraying the edges with "Nobecutane" (Astra Pharmaceuticals Ltd., Watford). The wounds had healed within a few days and mice were observed weekly for signs of growth of the implants. When moribund, the mice were killed and developing cysts were removed. They were divided into several pieces with one being placed in formol saline for histology and the remainder being chopped further for transfer to further mice or for EM examination.

RESULTS

RESULTS

1. Lymphocyte Proliferation Assays

1.1 Virus-derived Antigens

In preliminary studies, mononuclear cells from 15 patients showing varying degrees of CIN in cervical biopsies were examined in LPA using two BPV-derived antigens, namely, CsCl purified BPV (CsBPV at 30 µg/ml, 3 µg/ml and occasionally 0.3 µg/ml representing 10^{-1}, 10^{-2} and 10^{-3} dilutions of stock virus) and detergent-disrupted purified BPV (SDS.ME/BPV at similar concentrations). Cells were harvested at various times from 5-9 days after stimulation and the counts analysed as described in the Methods (Section 2.12). Table 7 shows that 8 of the 15 patients were positive on at least one time point with most positive on days 8 and 9; mean counts in wells with antigen varied considerably from patient to patient by this late stage and standard errors tended to be nearer 20% rather than the 10% generally considered acceptable. Nevertheless it was felt that weak positive reactions might be missed if cells were harvested too early and a compromise of 8 days after stimulation was set for further investigations with virus-derived antigens, although a few experiments were analysed at 6 and 7 days.

In an attempt to reduce the variations in counts in replicate wells and the wide range and variations within samples, cultures from 9 people were set up in duplicate with one set being "fed" an extra 100 ul of complete RPMI after 5 days. No improvement in variation in counts was noted.

1.11 Healthy Members of Staff

PBM from thirteen members of staff (11 female and 2 male) were mixed with various papilloma virion antigens in a standard LPA. Cells were harvested at 7 or 8 days after stimulation. The results (Table 8) show that 4 people (30.8%) responded to PV antigens. Both Subjects 1 and 2 had small finger warts which were resolving at the time the positive specimen was taken; Subject 3 had had hand warts;

Table 7 Lymphoproliferative response to BPV antigens at various time points in preliminary experiments

Specimen	No. days in culture	30µg/ml CsB cpm ± SD ± SE	SI	3µg/ml CsB cpm ± SD ± SE	SI	30µg/ml SDS.ME B cpm ± SD ± SE	SI	3µg/ml SDS.ME B cpm ± SD ± SE	SI	No antigen cpm ± SD ± SE
85567	5	653 ± 98 ± 56	0.7	731 ± 21 ± 12	0.8	421 ± 183 ± 106	0.5	616 ± 108 ± 62	0.7	915 ± 260 ± 150
	8	2499 ± 665 ± 384	**4.0**	1736 ± 415 ± 239	**2.8**	2506 ± 547 ± 316	4.0	3869 ±1161 ± 580	6.1	630 ± 80 ± 47
85568	5	356 ± 75 ± 43	0.9	300 ± 59 ± 34	0.8	238 ± 33 ± 18	0.6	277 ± 69 ± 40	0.7	395 ± 122 ± 87
	8	410 ± 134 ± 77	1.1	499 ± 24 ± 14	1.3	1712 ±1520 ± 878	**4.3**	424 ±2547 ±1472	**10.7**	375 ± 264 ± 152
85576	6	866 ± 131 ± 75	0.8	420 ± 347 ± 200	0.9	734 ± 83 ± 48	0.7	677 ± 112 ± 64	0.6	1078 ± 153 ± 88
	7	1144 ± 242 ± 139	0.7	1929 ±1142 ± 600	1.2	838 ± 539 ± 311	0.5	1912 ± 941 ± 513	1.2	1662 ± 552 ± 394
	8	3862 ±2815 ±1627	**2.5**			1618 ± 909 ± 525	1.1			1541 ± 596 ± 344
85581	6	456 ± 123 ± 71	0.7	603 ± 41 ± 23	0.9	553 ± 150 ± 86	0.9	522 ± 205 ± 118	0.8	640 ± 210 ± 121
	7	537 ± 278 ± 160	**3.0**	642 ± 408 ± 235	**3.5**	349 ± 98 ± 56	1.9	311 ± 79 ± 46	1.7	183 ± 32 ± 18
	8	451 ± 154 ± 89	**2.8**			359 ± 141 ± 81	**2.2**			162 ± 21 ± 12
85584	8	760 ± 101 ± 58	0.8			670 ± 129 ± 75	0.7			996 ± 191 ± 109
	9	1318 ± 544 ± 314	**3.5**			1338 ± 798 ± 460	**3.6**			373 ± 502 ± 290
85575	6	790 ± 269 ± 155	1.0	694 ± 116 ± 67	0.9	537 ± 218 ± 126	0.7	539 ± 297 ± 171	0.6	794 ± 111 ± 79
85580	6	603 ± 38 ± 27	0.9	792 ± 195 ± 139	1.2	466 ± 122 ± 70	0.7	742 ± 219 ± 126	1.1	674 ± 28 ± 16
85585	8	255 ± 9 ± 5	0.8			306 ± 32 ± 18	0.9			340 ± 16 ± 9
85598	8	1054 ± 404 ± 202	**2.1**	2333 ± 725 ± 363	**4.6**	295 ± 40 ± 23	0.6	1562 ± 134 ± 77	**3.0**	510 ± 64 ± 37
	9	1730 ± 323 ± 162	**2.0**	2169 ± 706 ± 353	**2.5**	310 ± 58 ± 29	0.4	2987 ± 359 ± 179	**3.3**	885 ± 30 ± 15
85599	8	479 ± 133 ± 67	0.5	678 ± 110 ± 55	0.7	94 ± 10 ± 5	0.1	584 ± 106 ± 53	0.6	922 ± 345 ± 173
	9	574 ± 119 ± 90	1.3	621 ± 246 ± 123	1.4	1027 ± 259 ± 130	**2.2**	980 ± 355 ± 177	**2.1**	459 ± 106 ± 53
85613	7	272 ± 23 ± 13	1.3			167 ± 26 ± 13	0.8			209 ± 109 ± 63
	9	388 ± 62 ± 35	1.0			113 ± 9 ± 5	0.4			319 ± 78 ± 45
85614	7	144 ± 20 ± 10	0.7			95 ± 17 ± 9	0.4			221 ± 32 ± 16
85615	7	190 ± 24 ± 12	0.9*			121 ± 11 ± 5	0.6*			207 ± 30 ± 15
85625	6	151 ± 23 ± 13	0.7*	146 ± 34 ± 20	0.7+	225 ± 51 ± 30	1.0*	246 ± 88 ± 51	1.1+	224 ± 18 ± 10
	8	428 ± 111 ± 64	1.1*	492 ± 21 ± 12	1.3+	458 ± 90 ± 52	1.2*	386 ± 18 ± 10	1.0+	390 ± 116 ± 67
85626	6	770 ± 68 ± 40	**2.4***	544 ± 210 ± 121	1.7+	544 ± 13 ± 8	1.7*	572 ± 71 ± 41	1.8+	321 ± 78 ± 45
	8	2382 ± 839 ± 485	**7.4**	3945 ±2047 ±1183	**12.3+**	2502 ±1187 ± 686	**8.7**	4228 ± 32 ± 18	**13.2+**	321 ± 132 ± 76

Notes * = 3 µg/ml antigen
+ = 0.3 µg/ml antigen

Summary of results:

Results	5 days	6 days	7 days	8 days	9 days
Positive	0/2	1/6	1/5	6/10	2/4
Negative	2/2	5/6	4/5	4/10	2/4

Abbreviations for antigens used in this and subsequent tables:
CsB, CsH1, CsH2 = caesium chloride purified BPV, HPV-1 or HPV-2
SDS.ME B = purified BPV disrupted by 2% SDS + 2% ME
gly B, gly H2 = glycine extracted BPV or HPV-2
unp H1 = unpurified HPV-1

Table 8 Lymphoproliferative response to PV antigens after 7-8 days in culture in health laboratory personnel, six of whom were tested on more than one occasion (subjects 1-6)

Subject	Specimen	Response with no antigen			Response with Antigen, expressed as S.I.															
		cpm	SD	SE	CsB Conc	CsB S.I	SDS.ME B Conc	SDS.ME B S.I	gly B Conc	gly B S.I	CsH1 Conc	CsH1 S.I.	SDS.ME H1 Conc	SDS.ME H1 S.I	unp H1 Conc	unp H1 S.I.	CsH2 Conc	CsH2 S.I.	SDS.ME H2 Conc	SDS.ME H2 S.I.	gly H2 Conc	gly H2 S.I.
1	A	417	± 42	± 24	3	1.1			4	1.5					7	**4.3**					9	1.9
					0.3	**2.2**			0.4	**2.1**					0.7	1.3					0.9	1.5
	B	1236	± 187	± 76							8	1.3	4	1.8	7	1.1						
	C	803	± 187	± 93							8	0.6	4	1.4	7	1.0						
	D*	2049	± 627	± 369											7	1.1					9	1.0
	E+																					
	F+	1743	±1349	± 780			1.5	0.4	40	0.4			4	0.3	70	0.3			1	0.2	90	0.4
									4	1.1					7	0.5					9	0.5
2	A	1388	± 516	± 298	3	0.9			4	0.8					7	1.0					9	1.3
					0.3	1.5			0.4	1.1					0.7	1.1					0.9	1.5
	B*	1223	± 229	± 132											7	1.1					9	1.3
	C																					
	D×	8348	±3195	± 1868	3	1.0	1.5	0.6	40	**4.3**	8	0.4	4	0.3	70	**4.6**	2	0.6	1	0.6	90	**4.0**
									4	**3.6**					7	**4.3**					9	**3.5**
3	A	1177	± 745	± 373	30	1.0			40	0.6					70	**2.2**					90	**2.1**
					3	1.1			4	0.9					7	1.7					9	1.1
					0.3	1.3			0.4	0.4					0.7	1.0					0.9	0.8
	B	4148	±1458	± 843													2	1.7				
	C	1809	± 765	± 442													2	1.1				
	D	2971	± 632	± 369											7	0.7	2	0.9				
4	A	283	± 60	± 34	30	0.6			40	0.8					70	1.3					90	1.1
																					9	0.9
	B	971	± 282	± 163													2	**3.8**				
	C	324	± 36	± 21											7	1.1	2	0.9				
5	A*																					
	B*																					
	C	183	± 16	± 10													2	1.0				
	D	168	± 16	± 9											7	0.9	2	0.8				
6	A	982	± 16	± 10													2	1.6				
	B	157	± 77	± 44													2	1.4				
7	A	365	± 71	± 41											7	1.2	2	1.4				
8	A	297	± 18	± 11											7	1.1	2	1.2				
9	A	487	±219	± 127													2	1.5				
10	A	257	± 50	± 29													2	0.9				
11	A	1360	±106	± 61													2	0.9				
12	A	1801	±455	± 266											7	0.5	2	0.7				
13	A	983	±157	± 91											7	0.8	2	0.7				

0 = in µg/ml

* = used in other experiments (vide infra)

\+ = Flu-like illness developed a few days later

x = foetal calf serum used instead of autologous plasma in medium

and Subject 4 had a tiny wart on the thumb which had appeared and disappeared over several years. It was possible to test PBM from Subjects 1 and 2 on several occasions and link the positive response obtained to the presence of their small warts (Fig. 11). Such a correlation was not possible in Subjects 3 and 4.

When PBM from the same group of subjects were analysed for their reponsiveness to PV antigens at different times after in vitro culture, 4 people gave positive responses (Table 9). Like Subjects 2 and 4, Subject 1 had a tiny hand wart at the time of testing while Subject 8 had no knowledge of any wart infections. Most of the other members of the group, despite their negative responses, remembered having had skin warts in the past.

As a consequence of their positive responses, Subjects 1,2, 3 and 4 were used several times in subsequent experiments (vide infra).

1.12 Patients with Recalcitrant Warts

A group of 10 patients, who had mosaic-type common warts on their hands or feet of more than 12 months' duration and resistant to at least 6 months of treatment were recruited as part of a study on the possible enhancement of the immune response during treatment with inosine pranobex (Imunovir ^{TM}, Edwin Burgess Ltd; Princes Risborough). Heparinised blood was taken by Dr. E.C. Benton, Dept. of Dermatology on entry to the trial, after one month of placebo or inosine pranobex treatment, and again one month after treatment had stopped.

PBM from this group were stimulated with glycine-extracted HPV-2 and purified HPV-2, and stimulation indices (S.I.) were calculated after 6 days in culture. Table 10 shows that 5 patients (50%) responded to at least one concentration of one antigen on one occasion, but the stimulation indices were low (except for the first specimens of patients CB4 and CB5). The pattern of response showed no correlation with the clinical state of their lesions. By two months, patients CB 1, 2, 8 and 9 were all showing improvement in their warts whereas CB 3, 4, 5, 6

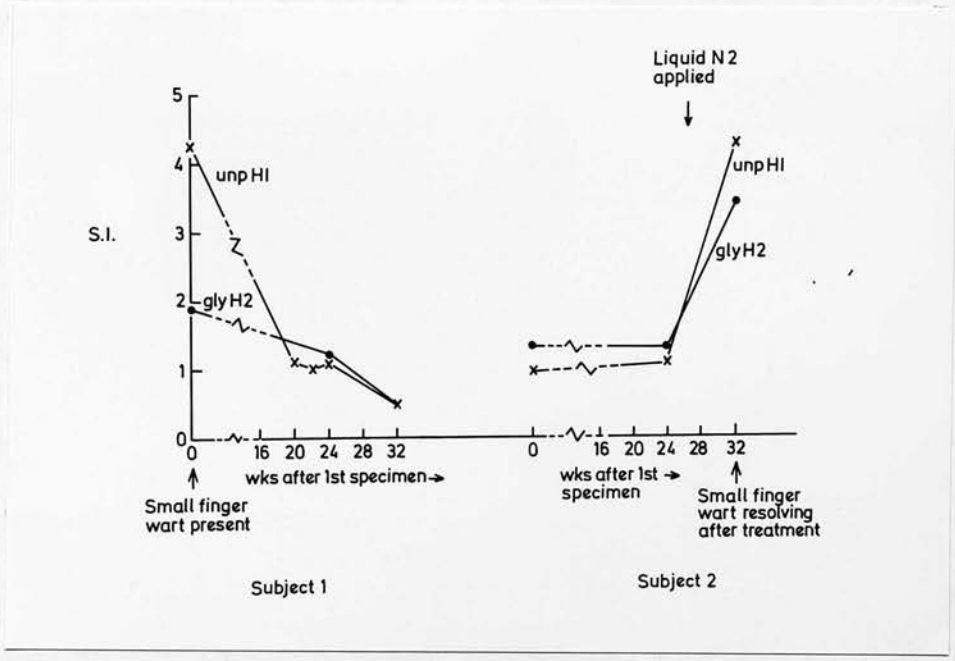


Fig 11 Lymphoproliferative response to skin wart antigens in two healthy members of staff around the time of infection

Table 9 Lymphoproliferative response to PV antigens in healthy laboratory personnel at various time points

Subject	Specimen No.	Antigen	S.I. after various days in culture		
			3-4 days	5-6 days	7-8 days
1	A	unp H1	.	1.1	1.1
		gly H2	.	1.2	1.0
2	B	unp H1	.	**2.5**	1.1
		gly H2	.	**2.3**	1.3
3	B	Cs H2	1.0	0.9	1.1
	C	Cs H2	1.0	.	1.1
	D	Cs H1	1.2	1.2	0.7
		Cs H2	1.1	0.8	0.9
4	B	Cs H2	**14.0**	**4.5**	**3.8**
	C	Cs H1	0.7	1.1	1.1
		Cs H2	0.7	0.9	0.9
5	C	Cs H2	1.4	0.7	0.9
	D	Cs H1	.	0.4	0.9
		Cs H2	.	0.4	0.8
6	A	Cs H2	1.0	0.9	1.6
	B	Cs H2	1.0	.	1.4
7	A	Cs H1	1.5	0.7	1.2
		Cs H2	0.8	0.9	1.4
8	A	Cs H1	**2.7**	0.8	1.1
		Cs H2	1.6	0.7	1.2
9	A	Cs H2	1.0	1.5	1.5
10	A	Cs H2	1.2	0.8	0.9
11	A	Cs H2	0.6	0.6	0.9
12	A	Cs H1	1.2	1.0	0.5
		Cs H2	1.0	1.0	0.7
13	A	Cs H1	1.6	1.6	0.8
		Cs H2	1.4	**3.1**	0.7

Table 10 Lymphoproliferative response to HPV-2 antigens in patients with recalcitrant common warts, 6 days after stimulation

Patient No.	Specimen 1 (at entry)						Specimen 2 (+ 1 month)						Specimen 3 (+ 2 months)						Specimen 4 (+> 3 months)					
	glyH2*			CsH2+			glyH2			CsH2			gly H2			C2H2			gly H2			C2H2		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
CB1	1.2	1.1	1.0		.		1.5	1.2	1.1	1.9	1.1	1.0		.			.			.			.	
CB2	**2.5**	1.2	.		.		1.1	0.7	0.9	.	0.9	0.7	0.7	0.7	0.7		.			.			.	
CB3	0.9	0.7	0.8		.		0.9	1.1	1.4	1.2	1.2	1.0	**2.1**	1.1	1.9	1.1	**2.1**	1.9	.	0.5	.	1.2	1.0	.
CB4	**11.8**	**13.5**	**12.5**		.		1.6	1.2	1.6	**2.3**	1.5	**2.1**	1.3	0.9	1.2	0.4	0.6	0.9			.		.	
CB5	1.1	1.2	**10.8**		.		1.2	1.4	1.1		.		1.1	1.5	0.8		.				.		.	
CB6	0.6	.	0.5	0.3	0.5	0.6	0.9	0.7	1.1	1.1	1.0	0.7	1.2	0.6	0.5	0.7	0.8	1.4			.		.	
CB7	0.5	0.6	1.0	0.6	0.8	0.5	0.5	0.8	0.6	0.5	1.2	0.7		.			.				.		.	
CB8	0.9	1.0	1.1	0.9	1.0	0.9	0.9	0.6	.	0.5	0.6	.	1.5	1.0	1.4	1.1	0.8	0.9	0.7	1.9	1.3	1.1	0.6	1.0
CB9	0.6	0.8	0.6	.	0.9	1.1		.			.		0.7	0.8	1.6	1.0	0.5	0.4	0.4	0.6	0.6	0.6	1.0	0.8
CB10	1.4	1.8	1.6	**2.0**	**3.5**	**2.1**	0.5	0.5	.	0.8	1.6	.	**2.8**	1.7	1.5	0.6	0.6	.		.			.	

* 10^{-1}, 10^{-2}, 10^{-3} gly H2 = 90, 9, 0.9 µg/ml

\+ 10^{-1}, 10^{-2}, 10^{-3} CsH2 = 40, 4, 0.4 µg/ml

and 10 showed no change (Table 11). Nevertheless, in the following month, patients CB 3, 8 and 9 were cured of their warts and interestingly all three had been given the active drug. Of the inosine pranobex-treated group only CB4 failed to show improvement, whereas in the placebo-treated group only CB2 and CB5 showed any clinical signs of improvement in their warts, as measured by a reduction in area covered by the lesions.

Experiments in the 1970's had suggested that healthy patients with long-standing warts have depressed CMI responses as measured by lymphocyte migration inhibition to non-specific mitogens (Morison, 1975c). The response of the "Imunovir Trial" patients to concanavalin A (Con A), phytohaemagglutinin (PHA), which are both T cell mitogens, and pokeweed mitogen (PWM), a B-cell stimulator, was therefore measured in an LPA assessed at 6 days and occasionally at 3 days. Table 12 shows that all patients responded positively at all stages to all mitogens tested. The variation in S.I. between different dilutions of mitogen and between specimens was considerable, but no evidence of a depressed non-specific CMI was found in these patients when lymphocyte stimulation was used as a measure, whether or not the patients were receiving active treatment. Although 6 days is likely to be beyond the peak response for non-specific mitogens and may account for the low T cell responses in patient CB4 specimen 3, and CB6 specimen 3, the few results obtained at 3 days do not all show a peak at this stage (CB8 specimen 2, CP10 specimen 2; Table 12).

When the treatment code was broken, it was evident that in the group receiving inosine pranobex, only patient CB4 failed to show improvement by the month after treatment, whereas in the placebo group, 4 out of 5 failed to show some improvement in the same period.

1.13 Patients attending the Colposcopy Clinic

PBM from 92 patients (including 11 of those described in Table 7) with a mean age of 33.8 years (±6.8 years) in whom CIN was

Table 11 Effect of treatment with inosine pranabex or Placebo in patients with recalcitrant common warts

Clinical appearance at each time point	+ 1 month (Specimen 2)	+ 2 months (Specimen 3)	+ 3 months	4-7 months	Treatment given
Cured	-	-	CB 3,8,9	CB 1,2,5,6	CB 1,3,8,9,- inosine pranabex CB 2,5,6 - Placebo
Improved	CB 2,3,(5),8	CB 1,2,8,9	CB 1,2,5	CB 7	CB 7 - Placebo
No change	CB 1,4,6,7,10	CB 3,4,5,6,10	CB 4,7,10	CB 4,10	CB 4 'inosine pranabex' CB 10 - Placebo
Worse	-	-	-	-	
Did not attend	CB 9	CB 7	CB 6	-	

Table 12 Lymphoproliferative response to T and B cells mitogens in patients with recalcitrant common warts

Patient	Specimen No.	Clinical Appearance of warts	S.I. - Con A 3µg/ml	S.I. - Con A 1µg/ml	S.I. - Con A 0.3µg/ml	S.I. - PHA 1:100	S.I. - PHA 1:400	S.I. - PWM 1:100	S.I. - PWM 1:400	Days in culture
A. Patients given inosine pranabex										
CB1	1	Plantar mosaics	30.6	22.5	16.4	34.2	36.7	58.7	55.3	6
	2	No change	20.1	34.2	19.7	20.3	30.1	28.4	27.5	6
CB3	1	Hand mosaics	35.9	14.6	10.6	25.6	31.4	38.2	49.1	6
	2	Dramatic improvement	14.8	36.2	31.0	28.6	32.0	23.7	33.8	6
	3	No further change	12.6	7.7	34.3	21.8	20.8	19.0	23.3	6
CB4	1	Foot & hand mosaics	41.5	27.0	18.6	59.6	54.7	28.8	45.0	6
	2	No change	17.5	48.2	49.4	46.2	26.6	27.3	43.1	6
	3	No change	7.9	4.4	2.4	6.4	7.1	12.4	14.3	6
CB8	1	Plantar mosaic	.	32.9	11.3	.	71.0	.	178.2	6
	2	Improved	.	3.5	.	.	77.3	.	23.6	3
			.	54.2	41.6	.	32.3	.	115.6	6
	3	Improved	.	44.3	6.6	.	152.5	.	258.4	6
	4	Clear	57.7	48.7	9.1	27.5	16.8	105.5	106.2	6
CB9	1	Foot & hand mosaics	.	40.7	21.2	.	35.5	.	99.1	6
	3	Improved	.	19.8	11.8	.	29.1	.	60.5	6
	4	Clear	16.6	19.7	11.6	3.3	5.6	17.9	16.7	6
B. Patients given Placebo										
CB2	1	Plantar mosaics	42.2	5.0	29.7	65.4	51.6	4.4	3.9	6
	2	Improved	49.6	76.8	28.2	44.1	60.9	41.3	44.8	6
	3	Improved	2.8	2.2	7.9	4.3	5.2	4.1	4.8	6
CB5	1	Hand & foot mosaics	27.1	21.4	10.1	11.3	25.6	38.4	42.9	6
	2	Slight improvement	56.2	65.6	26.0	87.0	93.3	36.6	50.5	6
	3	No improvement	7.8	9.8	17.2	16.9	19.0	13.0	13.9	6
CB6	1	Foot & hand mosaics	.	8.9	5.7	.	11.6	.	42.2	6
	2	No change	.	29.9	.	.	177.6	.	22.7	3
			.	56.0	59.9	.	18.4	.	65.8	6
	3	No change	.	6.0	5.0	.	6.7	.	26.4	6
CB7	1	Plantar mosaic	.	40.1	20.9	.	15.9	.	98.6	6
	2	No change	.	17.8	.	.	93.5	.	28.4	3
			.	58.0	34.7	.	14.8	.	65.9	6
CB10	1	Foot mosaics	.	59.8	30.4	.	63.6	.	141.2	6
	2	No change	.	5.68	.	.	16.7	.	12.9	3
			.	44.7	36.0	.	16.5	.	92.0	6
	3	No change	.	31.7	27.7	.	58.2	.	102.1	6

diagnosed at the time of sampling or had been present in the preceding year or two, were tested in an 8-day LPA using BPV, HPV-1 and HPV-2 antigens. Of the 92 patients, 23 gave positive S.I.s with at least one of the antigens and at one or more concentrations of that antigen.
The detailed analyses for the positive responders are given in Table 13. From the data presented, it can be seen that the S.I.s vary considerably and that stimulation with an antigen from one type of PV was not always accompanied by stimulation with another. Furthermore, no single concentration of antigen was found to be optimal and it was necessary to try both a range of concentrations and a range of antigens in order to obtain positive results. No positive S.I.s to bovine skin antigens were obtained. In Table 7 and Table 13 the mean cpm, standard deviation and standard error are all included for completeness but in subsequent Tables only the mean cpm and SE are presented.

While more positive results were obtained with the purified antigens (19/69; 27.5% of those treated) than with SDS.ME disrupted antigens (6/31; 19.4%) or using unpurified virions (3/16; 18.8%) or glycine extract (4.27; 14.8%) there were a few patients who reacted only to disrupted antigens (e.g. patients 86574 and 85568 in Table 13) and not to the purified antigen.

The S.I.s are plotted in graph form in Fig. 12. This figure includes the several different antigenic preparations used for each virus and therefore shows the number of positive results rather than the number of positive patients. However, only the optimal concentration of a particular preparation for each patient is given.

Of the 23 positive responses, 14 came from patients with histological evidence of koilocytes and this represents 31.1% of the K+ group. Nine were from patients without koilocytes, with only 19.1% of the K- group giving positive responses. This difference is just significant ($X^2 = 3.94$; $p < 0.05$). Positive responses were obtained in patients with all grades of CIN, with no significant difference between the number of each group ($X^2 = 4.58$; $p < 0.5$). (Table 14).

Table 13 Positive lymphoproliferative responses after 8 days in culture in patients with varying degrees of CIN

Specimen	Histology	BPV antigen						HPV-1 Antigen						No antigen		
		Conc. in µg/ml	Type	cpm		SD	SE	SI	Conc. in µg/ml	type	cpm	SD	SE	SI	cpm	SD	SE
85567	III K+	3	Cs	1736 ±		415 ±	138	2.8							630 ±	80 ±	47
		3	SDS ME	3869 ±		1161 ±	580	6.1									
85568	III K+	3	SDS ME	4234 ±		2547 ±	849	10.7							375 ±	264 ±	152
		3	Cs	499 ±		24 ±	8	1.3									
85576	II K+	30	Cs	3862 ±		2815 ±	938	2.5							1541 ±	596 ±	344
85587	III K+	30	Cs	451 ±		154 ±	51	2.8							162 ±	21 ±	12
			SDS ME	359 ±		141 ±	47	2.2									
85587	II K+	3	Cs	2333 ±		725 ±	363	4.6							510 ±	64 ±	37
		3	SDS ME	1562 ±		134 ±	77	3.1									
85626	III K+	3	Cs	3945 ±		2047 ±	1183	12.3							321 ±	132 ±	76
		3	SDS ME	4228 ±		32 ±	18	13.2									
85672	I K-	3	Cs	1126 ±		31 ±	18	2.1	8	SDS ME	645 ±	130 ±	75	1.2	524 ±	125 ±	73
85673	II K-	3	Cs	1500 ±		200 ±	115	4.6	8	Cs	738 ±	567 ±	21	2.0	328 ±	77 ±	39
86024	N past K+	3	Cs	559 ±		368 ±	184	3.5	8	Cs	571 ±	246 ±	123	3.6	159 ±	21 ±	13
									8	SDS ME	346 ±	90 ±	45	2.2			
86042	II K+	30	Cs	1233 ±		258 ±	129	1.0	80	Cs	26282 ±	11640 ±	5820	21.8	1207 ±	294 ±	172
86044	N past K-	.03	Cs	765 ±		147 ±	73	0.9	.08	Cs	3361 ±	717 ±	358	4.0	834 ±	284 ±	167
86048	N past K-	.03	Cs	809 ±		313 ±	156	3.0	.08	Cs	774 ±	387 ±	193	2.9	270 ±	34 ±	17
86055	II K+	.3	Cs	1589 ±		338 ±	169	2.5	8	Cs	769 ±	278 ±	139	1.2	647 ±	163 ±	81
86067	III K+	.3	Cs	4107 ±		581 ±	237	3.0	.8	Cs	1574 ±	818 ±	473	1.0	1388 ±	201 ±	116
86160	II K+	.3	Cs	322 ±		154 ±	69	2.4	.08	Cs	568 ±	422 ±	244	4.6	124 ±	9 ±	5
86162	I K+	3	Cs	283 ±		52 ±	47	1.4	.8	Cs	454 ±	129 ±	92	2.2	205 ±	14 ±	8
86202	N past K+	3	Cs	1036 ±		432 ±	216	1.1	8	Cs	2857 ±	2316 ±	1158	2.9	986 ±	503 ±	252
										unp	2288 ±	1035 ±	517	2.3			
86203	II K+	3	Cs	926 ±		313 ±	156	1.0	.08	Cs	4896 ±	2839 ±	1420	5.4	900 ±	258 ±	129
									70	unp	1742 ±	537 ±	269	1.9			
86204	I K+	3	Cs	1484 ±		154 ±	77	1.8	8	Cs	2886 ±	1362 ±	681	3.6	809 ±	113 ±	56
									7	unp	3715 ±	2459 ±	1421	4.6			
86219	N past K-	30	Cs	1397 ±		710 ±	355	2.1	8	Cs	2901 ±	1637 ±	818	4.4	710 ±	310 ±	155
86517	I K+	3	Cs	3559 ±		773 ±	446	1.8	7	unp	7503 ±	1913 ±	1106	3.8	1999 ±	483 ±	279
		.4	gly	3239 ±		154 ±	89	1.2									
86527	N past K+ GW now	.4	gly	5423 ±		3272 ±	1460	2.1	.9	gly*	11267 ±	9990 ±	4460	4.5	2525 ±	769 ±	343
86574	II K+ GW now	.4	gly	2837 ±		1111 ±	555	3.8	.9	gly*	2027 ±	816 ±	408	2.7	741 ±	223 ±	111
		.3	Cs	1378 ±		585 ±	338	1.9	.8	Cs	985 ±	731 ±	365	1.3			

gly* = glycine extracted HPV-2

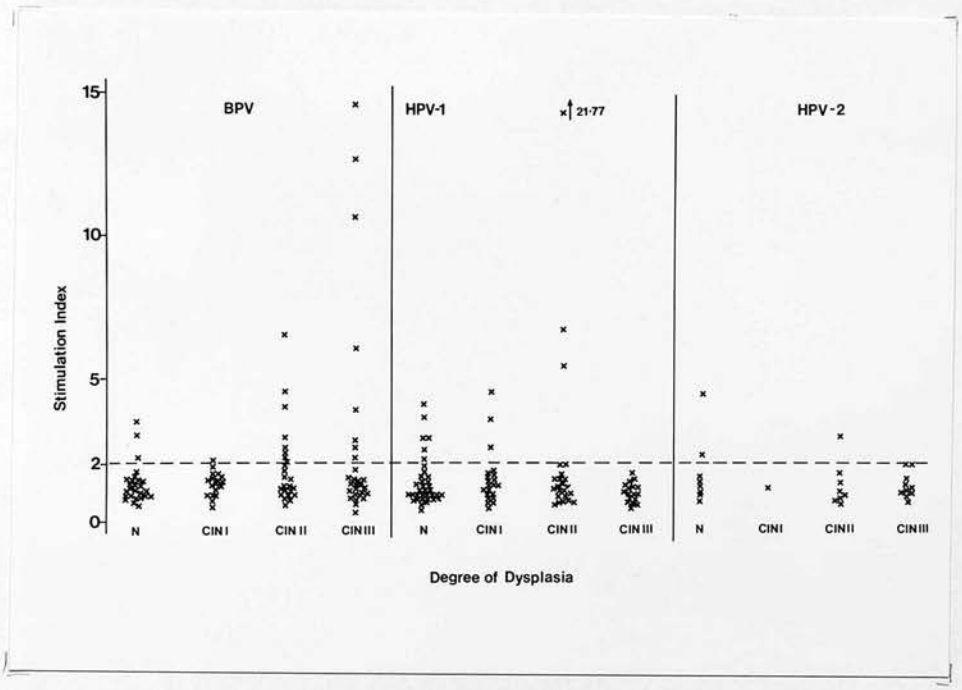


Fig 12 Lymphoproliferative response to PV antigens in patients with CIN

Table 14 The response of peripheral blood lymphocytes to papilloma antigens in patients with various degrees of CIN as shown by LPA

Grade of dysplasia	No of patients	No. with koilocytes (K+) or without (K-)	No. of patients with or without Koilocytes giving S.I. > 2.0	Total No. with S.I. > 2.0
No dysplasia at time of assay	28	13 K+ (past) 15 K- (past)	3 K+ 2 K-	5 (17.9%)
CIN I	14	7 K+ 7 K-	3 K+ 1 K-	4 (28.5%)
CIN II	22	14 K+ 8 K-	6 K+ 3 K-	9 (40.9%)
CIN III	28	11 K+ 17 K-	2 K+ 3 K-	5 (17.4%)
Totals	92	45 K+ 47 K-	14 K+ (31.1%) 9 K- (19.1%)	23 (25%)

Interestingly, while no correlation with koilocytosis was found, the positive cases came almost exclusively from those with a history of skin warts (Table 15). Out of 68 who knew they had or had had warts, 21 (30.9%) responded in the assay, compared with only 2/24 (8.3%) with no acknowledged history. This difference is highly significant ($X^2 = 10.42$; $p < 0.01$).

An analysis of 82 patients with CIN for whom full information was available showed that the youngest age group (20-29 years) contained the highest proportion of positive responder (7/21; Table 16). When information regarding smoking habits was examined, it was noted that 43% of smokers in the oldest age band (>40 years) responded to papilloma antigens despite the response rate in smokers of all ages being similar to that in non-smokers (Table 16). The number of patients in each group is too small, however, for these results to be significant.

A further 54 patients were examined during 1987 and 1988 with a similar range of antigens and lymphocyte proliferation was assessed at 6-8 days. Ten patients (18.5%) gave a positive response with eight of these responding to purified HPV-1 and HPV-2 including two who also responded to glycine extracted HPV-2. A further 2/30 responded only to unpurified HPV-1. All patients who responded had a past history of hand or plantar warts and indeed 3 had finger warts at the time of examination.

1.14 Summary of Results on Lymphocyte responsiveness to PV antigens

While 30% of healthy laboratory staff responded to PV antigens in vitro with positive results showing some correlation with the presence of unobtrusive hand warts, 50% of patients attending a Dermatology Clinic because of long-standing warts had low levels of memory T cells capable of responding in LPA. In this group however, no correlation was observed between a positive response and clinical improvement in their lesions. Contrary to earlier studies, these patients

Table 15 Influence of skin wart history on the outcome of LPA against papilloma antigens in patients with CIN

Skin wart history	No. of patients	No. with S.I. > 2.0	Presence (K+) or absence (K-) of koilocytes in positive responders
Past or present warts	68	21 (30.9%)	13 K+ 8 K-
No known history	24	2 (8.3%)	1 K+ 1 K-
Totals	92	23	14 K+ 9 K-

Table 16 Influence of age and smoking habits on the response to PV antigens in LPA in patients with CIN

Age range of patients	Smokers		Non-smokers		Total Positives
	Total No	Positive responders	Total No	Positive responders	
> 40 years	7	3 (43%)	6	0	3/13 (23%)
30-39 years	35	6 (17%)	13	3 (33%)	9/48 (19%)
20-29 years	12	4 (33%)	9	3 (33%)	7/21 (33%)
Totals	54	13 (24%)	28	6 (21%)	19/82 (23%)

did not show depressed LPA responses to non-specific mitogens. In a group of 92 Colposcopy Clinic patients, 25% responded to PV antigen(s) in LPA, a similar percentage to that found in staff members. While no correlation was found between positive responses and the degree of dysplasia nor between responsiveness and the presence of koilocytes, 91% of the responders had a history of past or present skin warts. It seems likely that the LPA assay was detecting exposure to common skin wart types rather than any association with cervical papilloma infection.

1.2 Cervical Tissue-derived Antigens

A glycine extract of autologous cervical tissue from 18 patients attending the Colposcopy Clinic and showing varying degrees of dysplasia was produced as described in Section 2.11 of the Materials and Methods. It was considered that such extracts might contain virally-induced cell-associated antigens. Normal tissue from a different area of the transformation zone was obtained in three patients. The protein concentrations of these extracts ranged from 3.5-90 µg/ml. It can be seen from Table 17 that only one of the eighteen responded positively to the glycine extract. The patient (87471) gave no other positive responses in this specimen but a second specimen 6 months later gave positive S.I.s in LPA with HPV-16E6, HPV-18E6 and HPV-16E4 fusion proteins as antigens (vide infra). In one patient (87121) there was a large reduction in response to the extracts of both "abnormal" and "normal" cervix, possibly due to a toxic effect of the extract. Because of the difficulties in obtaining matching bloods and tissue from histologically normal and abnormal areas, no further specimens could be examined in this way.

1.3 HPV Fusion Proteins

1.31 Fusion Proteins of Late and Early Genes of HPV-1 and HPV-2

Small amounts of L1, L2 and E2 fusion proteins of HPV-1 and of L2 of HPV-2 were available for testing in LPA. Fifteen

patients with varying degrees of CIN

Patient	Clinical Details	Conc. of protein in extract in µg/ml	S.I. in presence of cervical extract 1-10 µg/ml	0.1-0.99 µg/ml	Other responses
87120	CIN III, K+	88 (abnormal)	0.8	0.7	S.I. **9.3** to Cs H1
		90 (normal)	1.2	1.3	and **4.5** to gly H2
87121	CIN III, K+	88 (abnormal)	0.3	0.4	NPR
		68 (normal)	0.5	0.5	
87127	CIN III, K+	75	0.7	1.0	NPR
87129	Ca, K-	40	1.1	1.1	NPR
87177	CIN II, K+	15	0.9	0.7	NPR
87178	CIN I, K+	15	0.7	1.2	NPR
87183	CIN III, K-	25	1.4	1.2	NPR
87184	CIN III, K+	15	1.2	0.9	NPR
87195	CIN II, K+	4.5	0.8	0.9	NPR
87196	CIN III, K-	3.5	0.9	0.9	NPR
87197	CIN II, K+	4	1.2	1.2	NPR
87222		25	0.8	0.7	NPR

			Neat	10^{-1}	10^{-2}	10^{-3}	
87470	CIN I, K+	N.D.	0.9	0.9	0.8	1.1	NPR*
87471	No dysp. K- abnormal	N.D.	1.3	**2.1**	**2.5**	.	NPR
	normal	N.D.	1.0	0.5	0.7	1.7	.
87472	CIN III, K+	N.D.	0.4	0.3	0.6	0.4	NPR
87473	CIN II, K+	N.D.	1.9	1.75	0.9	.	NPR
87486	CIN III, K+	N.D.	0.3	0.6	.	.	NPR
87487	CIN II, K+	N.D.	.	1.9	.	.	NPR

NPR = no positive responses to papilloma virus antigens

* = specimen 6/12 later had positive reactions to HPV-16 and HPV-18 fusion proteins (See Tables 19 and 20).

specimens comprising 11 specimens from 5 patients with recalcitrant warts, one specimen from a laboratory member (Subject 2) and three specimens from colposcopy patients were tested with some or all of these antigens at concentrations adjusted to 3-30 μg/ml. Only one positive reaction to an HPV-1 fusion protein (HPV-1 L1) was noted, in a colposcopy patient who had had a plantar wart ten years before. Two positive responses to HPV-2 L2 were observed : in patient 87058 who had previously had cervial dysplasia without evidence of koilocytes and who had a wart on her thumb at the time of testing and in patient CB10 who had mosaic warts on both feet at the time of testing (Table 18). The few very low S.I.s obtained may reflect a toxic reaction to residual SDS in the antigen preparation.

Thus it can be seen that 3/8 patients responded to a late gene fusion protein and, despite the presence of contaminating β-galactosidase sequences, the results showed some agreement with the history of skin warts. The use of highly purified L1 or L2 fusion proteins might therefore be suitable for specific LPA testing against those HPV types which produce a complete replicative cycle. More work witih well defined groups of patients is required.

1.32 Fusion Proteins of Early Genes of HPV-16 and HPV-18

The lymphoproliferative response to HPV-16E6 and HPV-18E6 was assessed in 48 patients attending the Colposcopy Clinic (mean age 31.6 ± 7.0 years) and in 15 healthy female members of staff (mean age 30.1 ± 7.6 years), after 7 days in culture and at earlier time points in those patients from whom sufficient mononuclear cells were obtained. Against a background of culture medium as mock antigen 8 patients responded to one or both proteins (Fig. 13) but the responses were low with S.I.s in the range of 2.04-5.39 being recorded. None of the staff responded and none of the patients nor the staff showed positive responses at 3 or 5 days.

The lymphoproliferative response to a control protein similarly

Table 18 Lymphoproliferative response to fusion proteins of late genes of HPV-1 and HPV-2

Patient	Clinical details	No antigen cpm	No antigen SE	S.I. to HPV-1 L1 30µg/ml	HPV-1 L1 10µg/ml	HPV-1 L2 30µg/ml	HPV-1 L2 10µg/ml	HPV-1 E2 30ug/ml	HPV-1 E2 10µg/ml	HPV-2 L2 30µg/ml	HPV-2 L2 10µg/ml
87058	No dysplasia K-, PH+, thumb wart present	249	9	0.9	1.0	1.2	1.0	0.7	1.0	**29.4**	0.8
87059	No dysplasia K- finger wart present	1171	303	0.3	0.3	0.4	0.5	0.2	0.6	0.3	0.5
87063	CIN II K+, plantar wart 10 years ago	752	112	1.0	**7.3**	0.8	0.9	0.9		1.0	
Subject 2-E	Finger wart resolving	5110*	228	0.2		0.3		0.3		0.2	
CB6/1st spec.	Plantar mosaic and common hand warts for 2yr	1015	42							0.4	0.4
2nd spec.	No change	1142	207			0.4	0.4			0.7	0.9
3rd spec.	No change	1734	178							0.4	0.8
CB7/1st spec.	Plantar mosaic for 18 mths	457	102	0.7+	0.7	0.6+	0.8	0.7+	0.9	0.9+	0.7
2nd spec.	No change	1136	196			0.8	0.7			0.6	0.9
CB10/1st spec.	Plantar mosaics both feet	147	7								**3.1**
2nd spec.	No change	714	148							0.3	1.2
3rd spec.	No change	581	49							0.8	0.6
CB8/2nd spec.	Plantar mosaic, 2½ yrs	511	111							0.9	1.2
3rd spec.	Warts almost clear	321	20							1.0	0.8
CB9/3rd spec.	Plantar mosaics, common hand warts - clearing	1353	183							0.5	0.4

* 10% FCS in RPMI instread of more usual 15% autologous plasma. This may account for high count in the absence of antigen.

\+ 3µg/ml of antigen rather than 30µg/ml used in this patient

PH = past history of warts

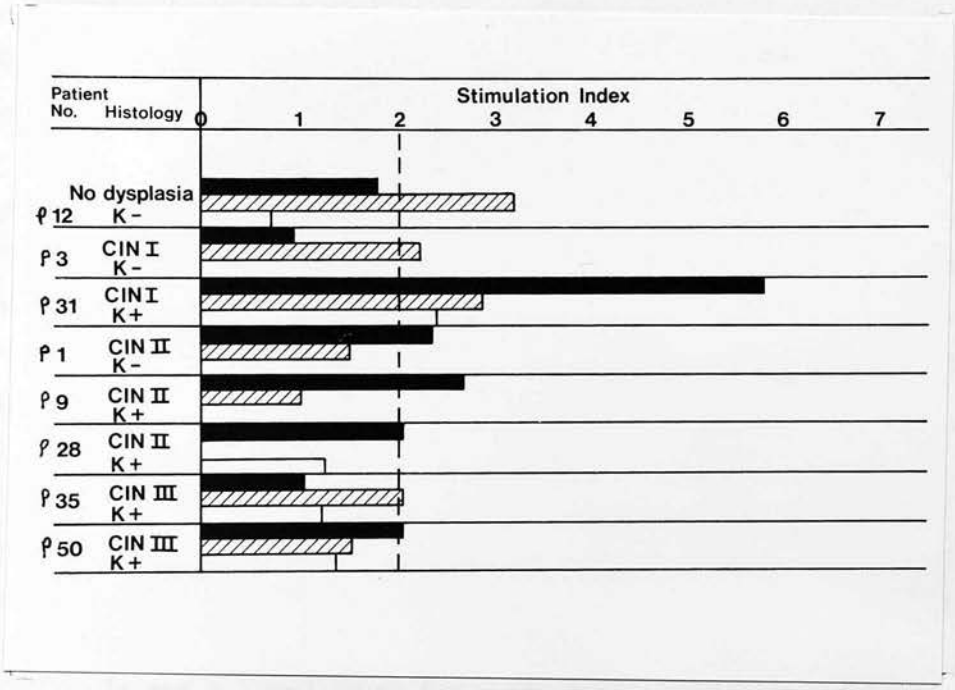


Fig.13 Lymphoproliferative response to HPV-16- E6 and HPV-18 E6 fusion protein in patients with CIN after 7 days in culture

HPV-16 E6 HPV-18 E6 Control E6

produced but lacking any HPV sequences was similarly assessed in 29 patients and all staff. Since only one patient and none of the staff responded (Table 19), the control protein was then used as a baseline for responsiveness to the specific fusion proteins. By this analysis, seven patients (24%) and three laboratory control (20%) responded to HPV-16E6 and/or HPV-18E6 after 7 days in culture. S.I.s were again low, ranging from 2.06 to 4.34 (Table 20). Only two patients (P12 and P31; Fig. 13 and Table 20) showed positive responses against both baselines.

Similar analyses were carried out using HPV-16E4-β-galactosidase fusion protein and a control β-galactosidase protein similarly produced. Against a background of culture medium PBM from two patients gave S.I. $\geq$2.0 to the HPV-specific fusion protein (Fig. 14). However responses to the control protein were obtained in five patients (Fig. 14) and additionally in one laboratory control after 7 days in culture and in another after 3 and 5 days in culture (Table 19). Against a baseline of the β-galactosidase response, PBM from only one patient showed an S.I. $>$ 2.0 to HPV-16E4 (P36; Table 20). The significance of the background responses to residual β-galactosidase is discussed.

It can be seen from Table 20, that patients who responded to fusion proteins in LPA presented with widely differing histological lesions. No correlation between S.I. and the degree of dysplasia nor the presence of koilocytes was obvious. Of the 48 patients, 33 had a past history of hand or foot warts and a further 7 had small common-type warts at the time of testing. Similarly in the control group, eight had had a previous skin wart infection and four currently had small warts. Six of 23 patients and 2 of 11 controls from whom sufficient PBM were obtained responded to purified HPV-2 or HPV-1 as antigens.

Table 19 **Lymphoproliferative response to control of HPV-16/18 E6 and HPV-16 E4 Beta-galactosidase fusion proteins in patients with CIN and in laboratory personnel**

Control protein	Patient No.	Days in culture	Lymphoproliferative Response to Culture medium x cpm	S.E.	Control prtoein x cpm	S.E.	(%)	S.I.
E6	P31	7			3087	748	(24)	2.37
E4-B-gal	P12*	7	328	8	835	111	(13)	2.55
	P26	7	473	71	1035	353	(34)	2.19
	P31	7	1305	298	5546	608	(11)	4.25
	P33	7	270	35	601	67	(11)	2.23
	P35	7	489	50	1447	174	(8)	2.96
	C5	7	706	113	1432	165	(11)	2.03
	C13	3	261	6	541	36	(6)	2.07
		5	540	51	1253	128	(10)	2.32

P = Patient
C = Control
x cpm = mean counts per minute
SE = standard error
SI = stimulation index
* = Patient 87471 in Table 17, PBM from whom responded to an extract of autologous cervical cells

Table 20 Lymphoproliferative response to HPV-16/18 E6 and HPV-16 E4 fusion proteins in patients with CIN and in laboratory personnel

Antigen	Patient No.	Histological findings	Days in culture	Lymphoproliferative response				
				Control protein		HPV protein		
				$\bar{x}$ cpm	SE	$\bar{x}$ cpm	SE(%)	S.I.
HPV-16E6	P12*	N,K-	7	224	17	581	88 (15)	2.60
	P31	I,K+	7	3087	748	7558	835 (11)	2.45
	P43	II,K+	7	1343	463	2911	637 (22)	2.17
	P44	CaCx	7	440	45	958	82 (9)	2.18
	P48	II,K+	7	503	65	1146	266 (23)	2.28
	C5		7	313	36	694	114 (16)	2.22
HPV-18E6	P36	N,K-	7	316	45	1373	366 (27)	4.34
	P43	II,K+	7	1343	463	3546	728 (19)	2.86
	P45	II,K+	7	1074	275	2215	186 (8)	2.06
	C2		7	240	46	656	89 (14)	2.73
	C5		5	350	86	928	58 (6)	2.65
			7	313	36	765	193 (25)	2.44
	C7		5	261	31	831	118 (14)	3.18
HPV-16E4	P36	N,K-	5	251	14	600	60 (10)	2.39
			7	527	155	1199	347 (29)	2.27

* = Patient 87471 in Table 17, PBM from whom responded to an extract of autologous cervical cells.

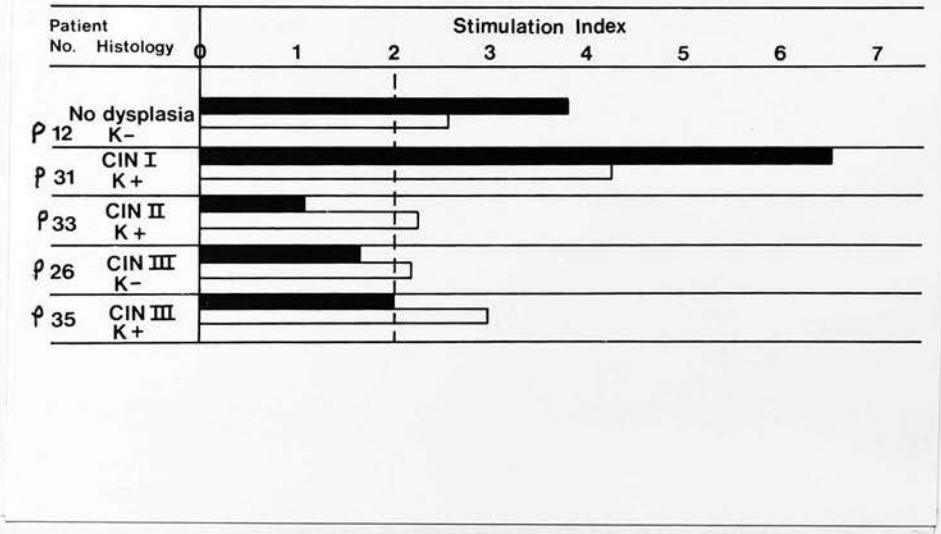


Fig 14 Lymphoproliferative response to HPV-16 E4 fusions protein in patients with CIN after 7 days in culture

HPV-16 E4 Control E4

1.4 Adaptations to the standard LPA

1.41 Addition of Lymphokines

The lymphokine IL-2 was added to proliferative assays with PBM from 68 specimens from 42 people. This included 32 specimens from 30 colposcopy patients, 26 specimens from the 10 patients with recalcitrant common warts and 10 specimens from 2 members of staff. By titrating the effect of IL-2 in the range 0.1%, 0.3%, 1%, 3% and 10% in samples from both members of staff and two colposcopy patients, it was found that 10% and 3% generally caused stimulation of the baseline 'Nil' response whereas concentrations less than 3% had little effect either on the baseline or on antigen-exposed cells. In subsequent investigations on the effect of IL-2 on responsiveness to PV antigens, concentrations of 3%, 5% and/or 10% were used. It can be seen from Table 21 that the addition of 10% IL-2 at 2-4 days or 3-5 % IL-2 on day 0 caused less background stimulation than addition of 10% IL-2 on day 0. However, the numbers of positive S.I.s obtained with PV antigens in the presence of IL-2 were not very different in each group when compared with the stimulation caused by IL-2 alone (Table 21).

The eleven specimens in which the presence of IL-2 produced an increased response with papilloma viral antigens are detailed in Table 22. Five specimens were from colposcopy patients and six were from people with cutaneous warts. No consistent patterns were obtained to suggest that addition of IL-2 on a regular basis would increase the sensitivity of detection of a weak proliferative response to papilloma antigens.

The second highest S.I. in the presence of IL-2 came from a colposcopy patient who had a thumb wart at the time of testing (87058). A positive response was also very obvious in the absence of IL-2 with the L2 fusion protein of HPV2 (see also section 1.31). Despite testing 19 specimens from colposcopy patients with HPV-16 and HPV-18 E6 fusion proteins and 10% IL-2, only one specimen (87485)

Table 21 Effect of addition of IL-2 on lymphoproliferation responses

% IL-2 added to culture	No. of specimens examined	Addition of IL-2 on day	No. of Pos S.I. in presence of IL-2 (%)	S.I. range in presence of IL-2 x S.I. ± S.D.	No. of Pos S.I. in presence of Ag + IL-2 (%)+
> 10%	27	0	20 (74.1)	4.1 ± 2.6	4 (14.8)
	13	2-4	6 (46.2)	2.1 ± 1.3	2 (15.4)
3-5%	32	0	13 (40.6)	3.4 ± 5.6 (2.2 ± 1.5)*	5 (15.6)

* excluding 2 S.I. of 15.5 and 31.6 which account for the hugh standard deviation when all 32 samples are assessed.

\+ considered positive when S.I. $\frac{\text{Ag + IL-2}}{\text{IL-2}} > 2$

Table 22 Positive lymphoproliferative responses to PV antigens in the presence of IL-2 at 7-8 days in 5 colposcopy patients and 6 people with skin warts

Specimen	Clinical Diagnosis	Response without Ag		IL-2 response				Antigen response							
		cpm	SE	Conc	Added on day	cpm	SE	SI	Conc Ag in µg/ml	without IL-2 x cpm	SE	SI	with IL-2 x cpm	SE	SI	S.I (Ag+IL-2)/IL2
86688	No dysplasia, past CIN III, PH+	108	3	10%	0	396	22	**3.7**	7 unpH1	162	29	1.5	1099	54	**10.2**	**2.8**
									2 CsH1	184	31	1.7	1215	94	**11.3**	**3.1**
86690	No dysplasia, past CIN III, PH+	244	110	10%	0	359	35	1.5	7 unpH1	156	8	0.6	903	64	**3.7**	**2.5**
									2 CsH1	136	25	0.6	1082	110	**4.4**	**3.0**
87017	CIN III, K+, PH-	489	90	10%	0	818	48	1.7	30 glyH2	388	7	0.8	16786	2424	**34.3**	**20.5**
87058	No dysplasia, K-PH+, HW present	249	9	10%	4	728	23	**2.9**	H2-L2*	7226	318	**29.0**	7930	101	**31.9**	**10.9**
87485[x]	CIN III, K+, PH+	778	135	10%	0	1059	86	1.4	6 18E6*	598	102	0.7	2207	130	**2.8**	**2.1**
88082[x]		443	28	10%	3	557	13	1.3	0.25 cE6	401	25	0.9	1649	144	**3.7**	**3.0**+
Subject 1-c	Recent hand wart	803	93	10%	0	501	75	0.6	2 CsH1	461	46	0.6	1027	76	1.3	**2.2**
									3 glyH2	639	89	0.8	1217	256	1.5	**2.4**
CB 1/2	Plantar mosaics	531	44	3%	0	832	79	1.6	3 glyH2	912	184	1.7	2308	217	**4.4**	**2.8**
CB 2/2	Regressing plantar mosaic	295	17	3%	0	325	115	1.1	3 glyH2	284	39	1.0	815	20	**2.8**	**2.5**
CB 7/1	Plantar mosaic	459	102	3%	0	1584	245	**3.5**	3 glyH2	286	30	0.6	8436	133	**18.5**	**5.3**
									0.3 glyH2	446	94	1.0	4942	817	**10.8**	**3.1**
CB 4/2	Plantar mosaic + common hand wrats	578	90	3%	0	1086	128	1.8	3 glyH2	700	15	1.2	3704	473	**6.4**	**3.4**
CB 10/3	Plantar mosaics	581	49	3%	0	386	71	0.7	4 CsH2	300	23	0.5	829	138	1.4	**2.2**
									30 glyH2	1691	88	**2.9**	538	92	0.9	1.4

* H2-L2 = HPV-2 L2 fusion protein; 18E6 = HPV 18E6 fusion protein

x Same patient six months apart

\+ analysed after 5 days in culture

gave a response with HPV-18E6 and IL-2 which was more than twice that of the IL-2 baseline. On several other occasions, IL-2 boosted the fusion protein response very slightly and in one or two specimens reduced it. Such reduction was also noted in 6 of the 68 specimens in this trial which had given S.I. $\geq$2.0 to a variety of papilloma antigens and which became negative in the presence of IL-2. In several of the colposcopy patients to whose cells IL-2 was added 2-4 days after E6 fusion proteins there was little response above background with one exception : an S.I of 3.0 was recorded with the E6 control protein plus IL-2 added at 3 days in specimen 88082 (repeat of 87485 six months later) but only when assessed after 5 days in culture.

Interleukin-1 (IL-1) was added at a dilution of 1 in 40 in RPMI to LPA from 3 specimens. In none of them was a positive response in the presence of IL-1 recorded. When added to a variety of PV antigens it caused no effect on the resulting S.I., although in Subject 1 (specimen C) it reduced the response to unpurified HPV-1 to half that obtained in the absence of IL-1 and in 86733 it similarly reduced the response to a glycine extract of HPV-2. Although IL-1 can promote T and B cell mitosis (Mims, 1987), this is not its main action and it was not considered worthwhile to add it to subsequent cultures.

1.42 Alteration in Number and Type of Antigen Presenting Cells

Attempts were made to alter the percentage of antigen presenting cells introduced into lymphocyte culture in the presence of antigen in the hope that more stimulation might be obtained. This was tried on 8 occasions with PBM from 7 people (6patients and 1 member of staff). Adherent cells were removed from PBM by adherence for up to 1 hour on glass (4 assays) or etched plastic (1 assay) with addition back into the non-adherent cell population of concentrations ranging from 1% - 10%. On three further occasions, not only were adherent cells removed on glass, but the non-adherent cells were enriched for T cells by passage through a nylon wool column. The

results, as presented, in Table 23 can be seen to be negative with one exception : specimen 86151 (patient with CIN III, K- and no known history of skin warts) gave strongly positive responses when adherent cells were added at a final concentration of 1% and 2% to cultures containing purified HPV-1 at three different concentrations. These results will be discussed.

Autologous cervical epithelial cells disaggregated from biopsy tissue by collagenase dispase digestion were added directly to PBM from 13 patients at a concentration of 4% (and also at 16% in specimen 86733). The results are summarised in Table 24. With two exceptions (86797 and 87017), the cervical cells had little effect on the baseline response with no added antigens, giving S.I.s in the range from 0.8 to 1.8. In specimen 87017 the cervical cells caused a reduction in S.I. to 40% of the baseline response, possibly suggesting a toxic effect after Percoll purification of the cervical cells resulting in a reduction of S.I. in response to unpurified HPV-1 and glycine-extracted HPV-2. Specimen 86797 (from a patient with no dysplasia) was the only specimen to give a positive response with cervical cells alone. It was also one of the two which gave a positive response to papilloma antigen alone (S.I. = 3.5 with unpurified HPV-1; specimen 86833 gave S.I. of 2.0 with same antigen; both specimens analysed after 6 days in culture). Nevertheless, the presence of cervical cells reduced the S.I. of HPV-1 containing wells to 68% of the index without cervical cells.

In two patients adherent cells were removed by attachment to glass for 1 hour and autologous cervical cells added back in place of them to the non-adherent population. In one case the non-adherent cells had been enriched for T cells by passage over nylon wool. In neither specimen was stimulation obtained with adherent or cervical cells in the presence or absence of papilloma antigens. However, the effectiveness of the depletion and reconstitution, for example by the alteration in Con A response, was not assessed.

Table 23 Addition of specific percentages of adherent cells to non-adherent cells in lymphoproliferative assays

Patient No.	Method of removal of adherent cells	T cell enrichment	% Adherent cells added to cultures	Response to PV antigens in Adh-LPA	Response to PV antigens in basic LPA
86148	60 mins on glass	-	3%	Neg	Neg
86151	60 mins on glass	-	3% 1%, 2%	Neg Pos	Neg Neg
Subject 1-C	15 mins on glass 30 mins on glass 60 mins on glass	- - -	3%, 10% 3%, 10% 3%, 10%	Neg Neg Neg	Neg but gly H2 Pos with IL-2 addition
Subject 1-E	45 mins on glass	-	1%, 3%, 10%	Neg	Neg
87115	60 mins on plastic	-	5%	Neg	Neg
86067	60 mins on glass	+	3%	Neg to Cs H1	Neg to Cs H1 Pos to Cs BPV
86241	60 mins on glass	+	5%	Neg	Neg
86324	60 mins on glass	+	0.3%, 1%, 3%	Neg	Neg

Table 24 The effect of adding cervical epithelial cells to LPA in the presence and absence of papilloma antigens

Specimen No.	Clinical Detials	Treatment of biopsy	S.I. in presence of 4% cervical (Cx) cells	Antigen response			
				Conc. in µg/ml	Ag	S.I. (without Cx cells)	S.I. (with Cx cells)
86596	III K- PH + Smoker 25/day	CDx2 hrs Washed	0.8	20 0.2 30	C2H1 CsH1 CsB	1.1 1.3 0.6	0.8 0.7 0.5
86605	CIN III K+ PH - Smoker 20/day	CD 2 hrs Washed	1.1	0.2 9 3 100	CsH1 glyH2 CsB B.S.+	0.9 0.9 0.9 0.7	1.0 1.1 0.9 0.6
86606	CIN II K- PH - Smoker 15/day	CD 2 hrs Washed	1.8	2 9 3 4 10	CsH1 GlyH2 CsB glyB B.S.+	1.6 1.3 1.1 1.1 1.7	1.4 0.9 1.3 1.0 1.4
86724	CIN II K- PH ? Smoker 20/day	CD 2 hrs washed	0.8	2 7 4 9 3	CsH1 unpH1 CsH2 glyH2 CsB	0.9 1.0 1.3 0.9 1.5	0.9 1.1 0.9 1.1 1.2
86732	CIN I K+ PH + Smoker	CD 2 hrs Washed	1.6	2 7 9	CsH1 unpH1 glyH2	1.2 1.4 1.3	1.5 1.8 1.6
86733	No dysplasia K-, PH + Non-smoker	CD 2 hrs Washed	4% 0.8 16% 1.0	2 7 9	CsH1 unpH1 glyH2	0.9 0.7 1.2	4% 0.6 16% 1.3 4% 1.4 16% 1.0 4% 1.1 16% 1.3
86796*	CIS K- PH +, non-smoker	CD 2 hrs Washed	1.1	7 9	unpH1 glyH2	1.3 1.2	1.3 1.4
86797*	No dysplasia	CD 2 hrs Washed	**2.3**	7	unpH1	**3.5**	**2.4**
86799*	CIS K- PH +, smoker	CD 2 hrs Washed	0.9	7	unpH1	1.2	0.9
86832*	CIN II, K+ HW present, smoker	CD 2 hrs Washed	0.8	7 9	unpH1 glyH2	1.2 0.9	0.9 0.7
86833*	CIN III, K+ PH +, non-smoker	CD 2 hrs Washed	0.8	7 9	unpH1 glyH2	**2.0** 1.0	1.5 1.1
87017	CIN III, K+ PH -	CD 2 hrs Percoll purified	0.4	7 9	unpH1 glyH2	0.7 0.7	0.4 0.4
87018	CIN III, K+ PH +	CD 2 hrs Percoll purified	Washer blocked	7 9	unpH1 x cpm glyH2 x cpm	378 323	x cpm 345 x cpm 281

* - harvested at 6 days

\+ BS - bovine skin

x CD = collagenase-dispase digestion

1.43 Attempts to Demonstrate a Suppressive Effect of PV Antigens

In addition to those patients who responded positively to PV antigens in LPA, a considerable number gave S.I. < 0.5 to one or more concentrations of one or more antigens tried: in 174 colposcopy patients tested with purified, unpurified or glycine extracted papilloma antigens, 37 (21.3%) gave reduced responses. (Any additional specimens giving reduced responses with SDS-disrupted antigens only were excluded because of possible toxicity from residual SDS); in 50 colposcopy patients tested with purified E6 fusion proteins, 17 (34%) gave reduced responses, with similar responses to E4 being excluded because of the possible confusion from the presence of B-galactosidase. Thus 54/224 (24.1%) gave S.I. < 0.5 to at least one PV antigen. The S.I.s ranged from 0.17 - 0.49 but the majority lay between 0.40 - 0.49. Supression of the response is subject to other influences on the immune system such as the effect of smoking. However, only 60% of those giving reduced responses admitted to smoking 2-30 cigarettes per day at the time of testing .

The reduction in S.I. was not considered to be a toxic effect of the antigens per se since many specimens gave non-remarkable results and approximately 25% gave positive responses. That both the positive results and the 'reduced' responses may be extremes of normal will be discussed. Nevertheless, it seemed possible that the reduced responses might be due to a suppressive effect and several experiments were carried out to try to elucidate this.

The effect of adding E6 fusion proteins to lymphocytes stimulated with Con A was examined at various times in 9 specimens from 6 members of the laboratory and 4 specimens from 4 colposcopy patients (Table 25). In the first group of seven specimens analysed at 7 days it was noted that in five the addition of high concentrations of purified E6 of HPV-16 or HPV-18 caused an increase in the S.I. compared with Con A alone (Table 25, Section A). This prompted a more thorough examination of the effect of HPV-16E6 and HPV-2 on

Table 25 Effect on lymphoproliferative response to Con A of adding PV antigens

	Specimen	Days in culture	No antigen x cpm	SE	S.I. to ConA	ConA + 16E6	ConA + 18E6	ConA + HPV-2	E6 fusion protein Conc. µg/ml	16	18	HPV-2 (µg/ml)	Effect of PV on ConA response: ConA +16E6 / ConA	ConA + 18E6 / ConA	ConA + HPV-2 / ConA
A	4-D	7	1262	143	**8.6**	**13.9**	**14.9**		25	0.7	0.7		1.6	1.6	
						8.8	**4.7**		2.5	0.3	0.3		1.0	0.6	
	4-E	7	408	112	**17.8**	**31.0**	**29.0**		25	1.4	1.1		1.7	1.6	
						25.7	**22.5**		2.5	1.6	1.1		1.4	1.3	
	5-A	7	877	50	**34.8**	**10.9**	**4.8**		2.5	0.8	0.8		0.3	0.1	
						13.9	**9.3**		0.25	0.5	0.8		0.4	0.3	
	5-B	7	349	93	**26.8**	**39.4**	**35.7**		2.5	0.8	0.7		1.5	1.3	
	87519	7	767	97	**6.2**	**15.5**	**14.7**		25	0.6	0.6		**2.5**	**2.4**	
						6.9	**8.4**		2.5	0.8	0.7		1.1	1.4	
	87920	7	521	48	**7.2**	**19.4**	**15.5**		25	0.5	0.5			**2.7**	**2.1**
						10.4	**11.2**		2.5	0.7	0.7			1.4	1.6
	87530	7	231	4	**117.3**	**86.0**			25	0.9				0.7	
						90.4			2.5	1.0				0.8	
B															
	4-F	4	209	28	**167.0**	**179.8**			25	**3.5**		**14.0**	1.1		
						200.1			2.5	**3.7**			1.2		
		5	339	43	**82.6**	**127.1**			25	**2.1**		1.6	1.5		
						104.4			25	**2.0**			1.3		
		6	351	75	**60.1**	**116.4**			25	**4.9**		**4.5**	1.9		
						96.1			2.5	**3.9**			1.6		
		7	734	231	**8.1**	**15.6**			25	0.5		1.5	1.9		
						11.4			2.5	0.5			1.4		
		8	971	163	**3.2**	**5.3**			25	0.7		**3.8**	1.7		
						5.1			2.5	1.1			1.6		
	3-B	3	500	20	**50.6**	**28.6**		**36.3**	25	0.9		1.0	0.6		0.7
		5	1273	117	**65.1**	**60.3**		**47.1**	25	1.6		0.9	0.9		0.7
		7	4148	843	1.1	1.6		1.0	25	**2.2**		1.7	1.5		0.9
	5-C	3	156	8	**11.1**	**10.1**		**7.4**	25	1.0		1.4	0.9		0.7
		5	237	36	**90.1**	**114.3**		**89.2**	25	1.1		0.7	1.3		1.0
		7	183	10	**53.1**	**94.1**		**94.1**	25	1.0		1.0	1.8		1.8
	6-A	3	306	6	**51.8**	**43.5**		**47.8**	25	1.1		1.0	0.8		0.9
		5	606	69	**70.9**	**97.9**		**91.6**	25	1.0		0.9	1.4		1.3
		7	982	10	**3.0**	**9.3**		**5.4**	25	0.9		1.6	**3.1**		1.8
	11-A	3	466	18	**18.1**	**10.0**		**19.0**	25	0.5		0.6	0.6		1.1
		5	740	47	**49.8**	**60.2**		**66.5**	25	0.6		0.6	1.2		1.3
		7	1360	61	**2.4**	**9.3**		**6.7**	25	0.4		0.9	**3.9**		**2.8**
	87599	3	135	9	**34.4**	**26.4**			25	1.9			0.8		
		5	346	58	**30.2**	**32.2**			25	1.0			1.1		
		7	1938	239	**2.5**	**3.6**			25	0.7			1.4		

the Con A response at various time points from 3-7 days. A progressive increase in response to Con A in the presence of PV antigen occurred over this time span in all six specimens tested (Table 25, Section B). Overall, in 4/13 (30.8%) specimens the S.I. with Con A was more than doubled by the addition of HPV-16E6 (specimens 87519, 87520, 6-A and 11-A) and in 1/4 (25%) it was more than doubled by the addition of HPV-2 (specimen 11-A). These results are shown graphically in Fig. 15. While the higher S.I.s at 7 days were thought to represent a shift in the Con A response due to an inhibitory effect of the PV antigen, the peak response (5 days) was not altered.

Because Con A is a non-specific mitogen of all T cells, it was considered appropriate to look for a similar effect in cells stimulated with HSV. UV-inactivated HSV was added at a multiplicity of 1pfu/cell and HPV-16E6 or purified HPV-2 was added in addition. The results from specimens from four laboratory members known to respond to HSV are shown in Fig. 16. No consistent pattern was obtained and any effect of PV antigen on the HSV response must be considered minimal. These results will be discussed.

To test whether a soluble factor was produced during incubation of PBM with PV antigens, supernate was collected from cultures of subject 4 at 4, 5 and 7 days after exposure to antigen. Subject 4 was chosen because of her previous positive responses to HPV-2 and HPV-16E6 (specimen 4-B, Tables 8 and 9; specimen 4-F, Table 25). Supernates were added to a subsequent specimen of PBM in culture on day 0 and the cells harvested on day 5. Unfortunately in this specimen, no positive response to HPV-2 or HPV-16E6 was recorded (S.I. of 0.93 and 0.78 respectively) and no effect was observed with any of the supernates. While this system is worth further study, the logistics of obtaining large enough volumes of blood on at least two occasions in close succession from people who had had previous positive results were daunting and no other specimens were treated in this way.

In order to assess whether the late 'delaying' effect on Con A

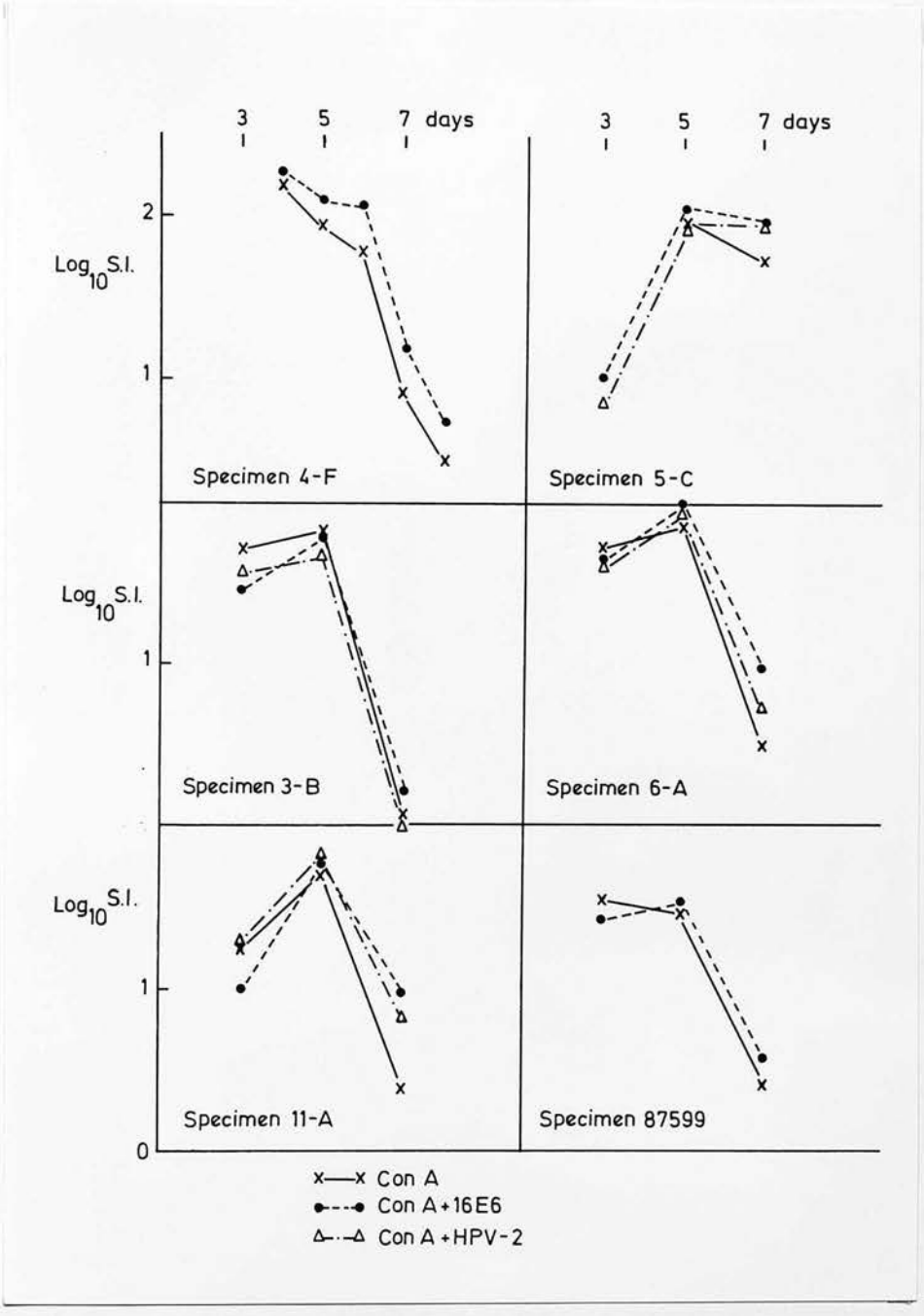


Fig 15 Alteration in lymphoproliferative response to Con A in the presence of HPV antigens

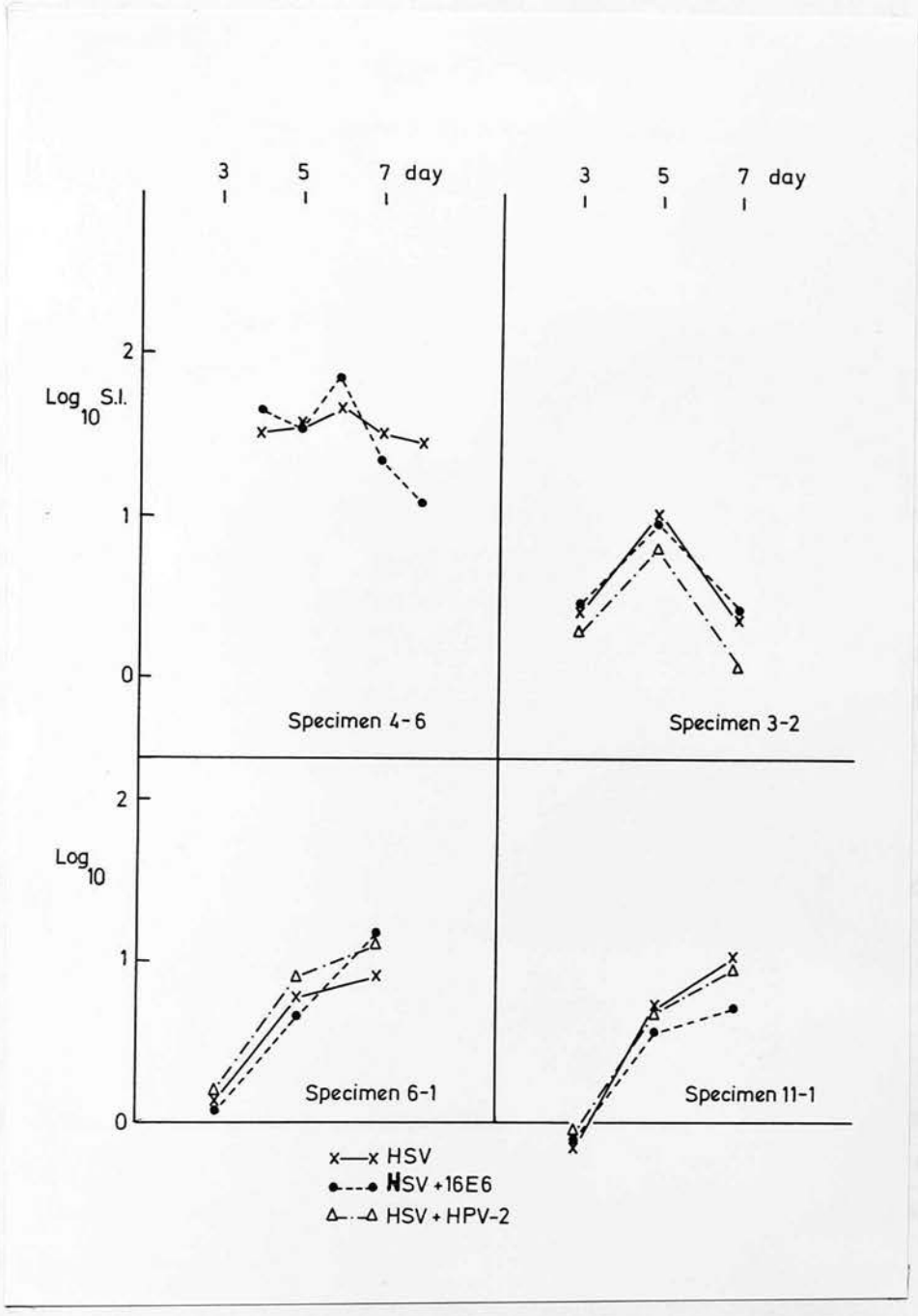


Fig 16 Effect of HPV antigens on lymphoproliferative response to HSV

responses and whether early positive responses (such as that shown by Subject 4, specimen F, Table 25) could be explained by the function of $CD8^+$ cells, these cells were removed from PBM by panning. Untreated PBM, $CD8^+$ depleted PBM and reconstituted PBM were all exposed to Con A, purified HPV-2 and HPV-16E6 fusion protein in a standard LPA assessed at 3 and 7 days in 3 people (Table 26).

Recent work in this laboratory has shown that panning is only partially successful in removing $CD8^+$ cells while cell sorting by EPICS analysis was considerably more effective. (Dr. James Vestey, personal communication). Nevertheless, it was found in all three patients that the removal of $CD8^+$ cells markedly reduced the Con A response at 3 days and their replacement at least partially restored it. This was the expected result for a non-specific T cell mitogen. By 7 days, the removal of $CD8^+$ with suppressor activity resulted in a maintained high S.I.

In the three people studied, reactions to PV antigens in whole PBM were minimal (with the exception of subject 6-B who showed a positive response to HPV-16E6 at 7 days). Nevertheless, partial removal of $CD8+$ cells reduced the HPV-16E6 response in Subject 3-C at 3 days to almost half and similarly in Subject 6-B at 7 days. The HPV-2 response in Subject 3-C was similarly reduced at 7 days. These results do not support adequately the suggestion that $CD8^+$ cells exerted a suppressive effect on the T cell response, although much more work with more efficient $CD8^+$ depletion in people who have been shown to respond positively to PV antigens is required.

1.44 Subset Analysis

Sufficient PBM were obtained from several specimens to attempt to investigate the effect of papilloma antigens on the percentage of lymphocyte subsets at various times after stimulation. Monoclonal antibodies to T1 and T3 as pan-T markers, T4, T8 and occasionally Leu 7 (NK cells) and anti-Tac (activated T cells) were used

Table 26 Effect on lymphoproliferative response to PV antigens of removal of CD8+ cells by panning

Specimen	Days in culture	Antigen Conc in µg/ml	Ag	S.I. Whole PMB cpm	SE	SI	$CD8^+$ depleted cpm	SE	SI	$CD8^+$ reconstituted (ratio 3:10) cpm	SE	SI
3-C	3		NIL	375	28		218	8		275	8	
		1	ConA	29367	1262	**79.4**	5770	470	**26.5**	12844	3017	**50.0**
		4	CsH2	360	23	1.0	216	18	1.0	280	23	1.1
		25	16E6	585	47	1.6	161	9	0.7	332	28	1.3
	7		NIL	1809	442		196	46		745	218	
		1	ConA	2965	107	1.6	23189	2201	**118.3**	8982	1010	**12.1**
		4	CsH2	2026	291	1.2	134	17	0.7	545	136	0.7
		25	16E6	908	65	0.5	123	17	0.6	279	28	0.4
6-B	3		NIL	265	10		107	21		127	11	
		1	ConA	9258	818	**34.9**	2116	535	**19.8**	8451	913	**66.5**
		4	CsH2	252	14	1.0	169	70	1.6	145	10	1.1
		25	16E6	274	31	1.0		.			.	
	7		NIL	157	44		53	6		81	6	
		1	ConA	13462	1006	**85.8**	16555	564	**312.4**	20159	278	**248.9**
		4	CsH2	220	24	1.4	90	11	1.7	70	3	0.9
		25	16E6	437	50	**2.8**	77	8	1.5		.	
88059	3		NIL	354	35		463	197		600	20	
		1	ConA	12544	2193	**32.7**	1005	100	**2.2**	634	35	1.06
		25	16E6	273	10	0.8	289	16	0.6	536	62	0.9
		25	18E6	254	12	0.7	315	44	0.7	533	97	0.9
		25	LE6	266	17	0.8	238	7	0.5		.	

in an indirect fluorescent assay in an EPICS cell sorter. B cells were stained directly with fluorescein-conjugated anti-human IgM raised in sheep.

The T1 marker gave exceedingly variable and low results, while on the few occasions on which T3 was used, 63±8% of the cells analysed were stained. In an investigation of T cell response to HSV antigens was carried out in this laboratory, the percentages of cell types staining with the same monoclonal were found to be 60±7% for T3, 44±10% for T4, 23±4% for T8 and 11±2% for B cells (Dr. James Vestey, personal communication). The percentages of cells stained in the current study were slightly lower and more variable particularly for the T4 marker. This may have been due to the storage of fixed cells after staining for up to 12 days before they could be analysed. Although freshly separated PBM should be robust, it appears that cultured cells are particularly fragile and storage after fixing leads to a loss of stain as well as disruption of the cells themselves.

As a result of the variation in staining, no conclusions could be drawn from the results. However it was interesting to note that in Subject 2-F (Table 27), despite positive S.I.s being obtained at 6 and 8 days with glycine-extracted HPV-2 and unpurified HPV-1 in the presence of IL-2, the percentage of T8 cells decreased with time suggesting that they were not involved in the proliferative response. On the other hand, when a specimen from Subject 4 who had previously shown positive responses to several papilloma antigens was analysed on days 4, 5 and 7 after exposure, an increase in T8 was noted on day 4 in the presence of purified HPV-2 and HPV-16 fusion protein, matching with positive S.I.s at this point. No similar increase in T8 was noted in the Nil control or in Con A stimulated cells. The percentage of T8 was reduced by day 5 in the presence of HPV-2, although it was still somewhat higher with HPV-16E6 and again matched with a positive but falling S.I. to HPV-16E6. By 7 days the percentages were comparable in each group of cultured cells. This encouraging result suggested that

Table 27 Lymphocyte subset analysis before and after culture in the presence of PV antigens

Specimen	Days in culture	Antigen Conc µg/ml	Ag	S.I.	T3 %	T4 %	T8 %	B %
2-F	0				.	34*	24	4
	6		NIL		.	42*	20	7
		9	gly H2	0.3	.	29*	19	5
		7	unp H1	0.7	.	29*	20	5
			10% IL-2	**6.2**	.	31*	19	2
		9	gly H2+IL-2	**10.7**	.	30*	20	5
		7	unp H1+IL-2	**3.6**	.	33*	20	5
	8		NIL		.		14	7
		9	gly H2	1.3	.	34*	12	10
		7	unp H1	1.3	.	34*	.	14
			10% IL-2	**3.6**	.	33*	7	8
		9	gly H2+IL-2	**2.7**	.	41*	8	11
		7	unp H1+IL-2	**4.0**	.	23*	9	9
4-F	0				.	31*	12	9
	3		NIL		.	20*	7	5
		1	ConA	**167.0**	.	19*	8	6
		4	CsH2	**14.0**	.	21*	21	8
		25	16E6	**3.5**	.	29*	33	.
	5		NIL		71	55	20	.
		1	ConA	**82.6**	60	55	18	.
		4	CsH2	1.6	72	56	16	.
		25	16E6	**2.1**	75	55	25	.
	7		NIL		59	43	14	11
		1	ConA	**8.1**	48	44	16	6
		4	CsH2	1.2	55	39	10	5
		25	16E6	0.5	61	43	15	17
88003	0				52	45	18	14
	2		NIL		60	63	25	13
		4	CsH2	1.1+	73	66	30	14
		0.25	16E6	1.1	66	48	9	13
		0.25	cE6	1.4	67	57	20	12

* = T4 percentages inexplicably low
\+ = S.I. to CsH2 at 7 days was **2.2**

analysis even before 4 days might be relevant and specimens from 5 colposcopy patients were therefore examined after 2 or 4 days in culture. In one patient a high T8 percentage was noted after 2 days in a culture stimulated with purified HPV-2. By 7 days the HPV-2 stimulated cells were showing an S.I. of 2.2 in LPA. Obviously these are preliminary results complicated by the variable staining patterns obtained with stored cells, but the use of the EPICS in analysis of this kind will be discussed.

Interestingly in several of the specimens examined after 2-3 days in culture, two populations of cells could be identified with each marker, on the basis of size differentiation. It was thought that the larger cells might represent activated cells and would stain preferentially with the anti-Tac marker. However, specimen 86003 which demonstrated these two populations appeared to have fewer anti-Tac positive cells distributed between the two cell types than specimens in which the cells were more homogeneous. The granularity of the larger cells was increased as might be expected in cells preparing for division.

1.45 Summary of Results of Adaptations to the Standard LPA

The addition of IL-2 to lymphocyte cultures in the presence of papilloma antigens, either structurally derived or bacterially produced fusion proteins, did not improve the sensitivity of the LPA. Addition at a later stage of culture appeared to be no more effective than addition at day 0. While positive responses in the presence of IL-2 were sometimes observed, the effect was not consistent and no obvious pattern appeared.

Alteration of the antigen presenting cells by increasing the concentration of adherent cells in the cell population failed to improve the sensitivity of the reaction. Similarly, adding cervical epithelial cells as potential APC or replacing the adherent cells with cervical cells failed to increase responsiveness.

In 25% of cases, PV antigens caused a depression rather than a stimulation, with S.I.s reduced to < 0.5. The addition of HPV-16E6 fusion proteins or HPV-2 appeared to potentiate the ConA response such that by 7 days the response had not fallen so far from the 5 day peak; this effect, however, was not so readily demonstrated by adding papilloma antigen to cells already mixed with HSV, nor was a soluble factor demonstrable at several time points after culture of a responder's PBM in various papilloma antigens.

A method for lymphocyte subset analysis using cells cultured and stained in microtitre plates was developed, and in two cases the results suggested that an increase in $CD8^+$ cells after 2-4 days in culture might be associated with the positive S.I.s in LPA.

Much work needs to be done in this area, but the greatest need firstly is to find consistently good responders in the basic LPA test.

1.5 T-Cell Lines

Six attempts to produce a line of cells specifically responsive to papilloma antigens were made when sufficient PBM were available after the standard LPA had been set up. When only a few million cells were available, they were set up at 2-3 x 10^6/ml in wells of a 24-well plate, and when more than 2 x 10^7 were available, Falcon flasks were used. Different antigens were tried for stimulating the bulk cultures.

Three specimens from colposcopy patients were set up, including one which responded to glycine-extracted HPV-2 especially in the presence of IL-2 (to give an S.I. of 34·83 with HPV-2 and IL-2), one which responded to the late fusion protein of HPV-1 (L1H1), and one which unfortunately failed to respond in LPA to the unpurified HPV-1 to which the excess cells were exposed. After 7-8 days in culture, bands of T lymphoblasts were obtained at the 40-50% interface

of a Percoll gradient, but none were successfully expanded in the presence of IL-2 and quickly died out.

Two specimens from patients in the inosine pranobex trial were similarly treated. One specimen from a patient who had rapidly resolving common hand warts was stimulated with purified HPV-2 while the other, whose mosaic hand and foot warts had not improved, was stimulated with glycine-extracted HPV-2, to which he had given a strongly positive response (S.I. = 13.54) one month earlier. Unfortunately, neither patient responded with a positive S.I. to these antigens at the same time as the cell-line cultures were started and no blasts were obtained when the cells were separated on a Percoll gradient.

The sixth specimen came from a member of staff (subject 2, specimen 4) and as shown on Table 8, she responded to several PV antigens at this time. Twenty million PBM in 10mls of complete RPMI were stimulated with 500µl of glycine extracted HPV-2 at a concentration of 10µg/ml. T-lymphoblasts were seen as two small bands at the 40-50% interface of a Percoll gradient after 7 days, and these were slowly expanded in culture over the next three weeks in the presence of 3% IL-2. They were then mixed with autologous irradiated PBM in the ratio of 10^5:10^6 and restimulated with the same antigen. However, when separated one week later on Percoll, no band of lymphoblasts was observed, the majority of cells were small and could not be maintained. Possibly a higher concentration of IL-2 would have allowed more rapid expansion in culture and given this developing cell-line a better chance.

As with adaptations to the standard LPA, it was realised that a good and regular responder had to be found first before trying again. It was easier to obtain a supply of autologous PBM for irradiating from laboratory members than patients, and with hindsight, subject 4 might have been suitable. However, even she did not respond consistently to any single PV antigen making it difficult to be sure of

the optimal antigen to use or its concentration.

2. Antibody Production

2.1 Plasma IgG against Virion Antigens

2.11 Healthy Members of Staff

Initially ELISAs were carried out using glycine-extracted BPV as coating antigen at a concentration of 4µg/ml, by the method described in Section 2.21 of the Methods. This produced strong positive reactions with rabbit antisera against SDS-disrupted BPV-1 from both a commercial supplier (Dako) and from a rabbit immunised in this Department. However, with human sera from laboratory personnel, absorbances were low and were never more than twice the background absorbance obtained with a glycine extract of normal bovine skin at a similar protein concentration.

Subsequently, using purified HPV-1 or HPV-2 as antigen, disrupted with 2% SDS and 2% ME and at a concentration of 1-2µg/ml, results were obtained which were considerably above the background absorbance obtained with an extract of human keratoses, enabling antibody positive and negative plasma specimens to be distinguished. A baseline was obtained using a plasma sample (86011) from a colposcopy patient with no history of skin warts, no cervical dysplasia nor koilocytosis, a negative response to various PV in LPA and who consistently gave very low absorbances on repeated testing. The cut-off was assessed on each run from the mean of at least three negative wells plus 3S.D., and titres were taken as the last dilution giving a higher absorbance than the cut-off.

The results of four titrations over a three month period on three members of staff using disrupted HPV-1 as antigen are shown in Fig. 17. It can be seen that in both subjects 1 and 2, the mean absorbance at a dilution of 1:3200 was still greater than the cut-off of 0.31 whereas in subject 3, the 1:50 dilution gave a mean absorbance very close to the cut-off. The estimated titres against HPV-1 and

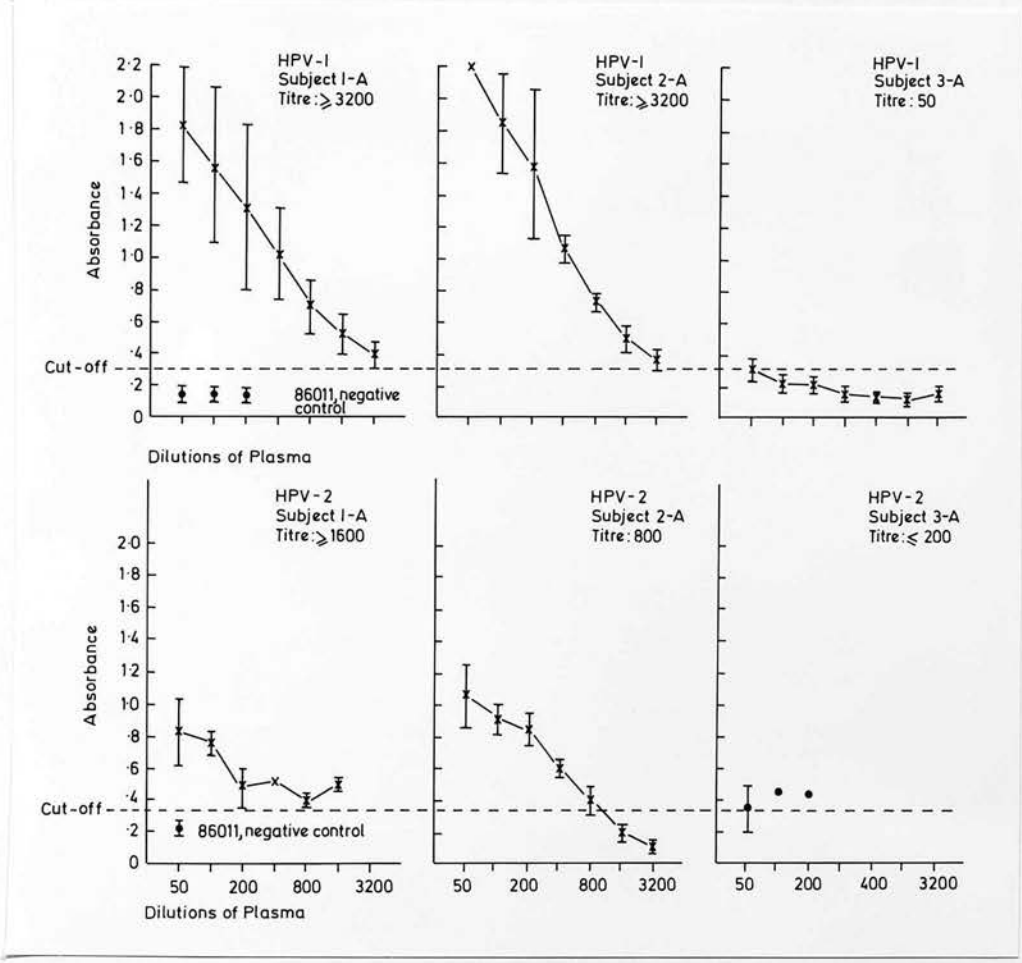


Fig 17 Antibody response to disrupted HPV-1 in three healthy members of staff measured by ELISA

HPV-2 in five laboratory members are shown in Table 28.

2.12 Patients with Recalcitrant Warts

Ten patients enrolled in the inosine pranobex described in Section 1.12 of the Results were tested for antibodies to HPV-2 at the time of enrolment in the study, after one month of treatment and after a further month without treatment. All specimens were tested at the same time. Only 4 of the 10 had antibodies at the time of entry into the trial and, no others had developed antibodies by the end (Table 29). Indeed, the borderline titres obtained in CB8 and CB9 in the early specimens were not detected in the final specimen. Only CB3 showed a boost in antibody titre and it is of interest to note that this patient received the active inosine pranobex. Although it took a further month for resolution of his multiple warts on hands and feet, there had been a dramatic clinical improvement between the first and second visit.

2.13 Colposcopy Patients

Plasma from 282 patients attending the Colposcopy Clinic were tested at a dilution of 1:50 for the presence of antibodies against purified SDS.ME disrupted HPV-1, HPV-2 and BPV in the standard indirect ELISA. ELISA Indices (E.I) were calculated from at least duplicate assays by dividing the absorbance of the test sample by the mean absorbance of at least three negative wells plus 3S.D. The E.I. allows some quantitative comparison of results obtained at a single screening dilution in different runs. One hundred and forty four (51.1%) were found to have antibodies (i.e., E.I. $\geq$ 1) to at least HPV-1 or HPV-2 with 73 (50% of the total) having antibodies to HPV-1 only, 64 (45.1%) to both HPV-1 and HPV-2 and only 7 (5.8% of the 139 tested) having antibodies to HPV-2 alone (Table 30). These results suggest that, despite SDS.ME disruption of virions, predominantly type-specific epitopes were being recognised by the humoral immune system.

Table 28 Antibody titres to HPV-1 and HPV-2 by ELISA in healthy laboratory staff

Subject - Specimen		History	Titre	
			HPV-1	HPV-2
1	A	Recent hand wart	> 3200	> 1600
2	A	Past plantar hand wart	> 3200	400
3	A	Past hand wart	50	200
4	A	Recurring tiny hand wart	< 50	200
5	A	Past plantar wart	> 200	< 50

Table 29 Antibodies to HPV-2 in 10 patients with recalcitrant common warts

Patient	Treatment (Active prep. or placebo)	1st Visit T-C at 1:50	1st Visit Titre	1st Visit T-C at this dilution	2nd Visit T-C at 1:50	2nd Visit Titre	2nd Visit T-C at this dilution	3rd Visit T-C at 1:50	3rd Visit Titre	3rd Visit T-C at this dilution
CB1	Active	.11	< 50	.	.11	< 50	.	.	NA	.
CB2	Placebo	.19	< 50	.	.21	< 50	.	.26	< 50	.
CB3	Active	.89	**200**	.73	.88	**200**	.53	1.22	**400/800**	.62/.40
CB4	Active	.23	< 50	.	.25	< 50	.	.22	< 50	.
CB5	Placebo	.25	< 50	.	.20	< 50	.	.26	< 50	.
CB6	Placebo	.19	< 50	.	.14	< 50	.	.12	< 50	.
CB7	Placebo	.76	**200**	.42	.77	**100**	.72		NA	
CB8	Active	.40	**< 50**	.40	.48	**50**	.48	.39	< 50	.
CB9	Active	.42	**50**	.42		NA		.35	< 50	.
CB10	Placebo	.22	< 50	.	.16	< 50	.	.16	< 50	.
Negative Control		.36 $\pm$ 0.02								
Cut-off		.42								

NA = not available

T-C = Absorbance of test minus absorbance of control well at 410 nm.

Table 30 Antibody response to HPV-1 and HPV-2 measured by ELISA in patients with various degrees of CIN.

Grade of dysplasia	No. of patients	No. with koilocytes (K+) or without (K-)	No. of patients with or without K giving E.I > 1.00	Total No. of patients with E.I. > 1.00 (%)	Positive responders to different HPV types
No dysplasia at time of assay	117	45 K+ (past) 72 K- (past)	22 K+ (48.9%) 38 K- (52.8%)	60 (51.3)	30 Type 1 only 2 Type 2 only 28 Both types
CIN I	39	23 K+ 16 K-	15 K+ (65.2%) 6 K- (37.5%)	21 (53.8)	9 Type 1 only 0 Type 2 only 12 Both types
CIN II	42	26 K+ 16 K-	14 K+ (53.8%) 8 K- (50.0%)	22 (52.4)	10 Type 1 only 1 Type 2 only 11 Both types
CIN III	84	36 K+ 48 K-	17 K+ (47.2%) 24 K- (50.0%)	41 (48.8)	24 Type 1 only 4 Type 2 only 13 Both types
TOTALS	282	130 K+ 152 K-	68 K+ (52.3%) 76 K- (50.0%)	144 (51.1)	73 Type 1 only 7 Type 2 only 64 Both types

No correlation was found between the presence of HPV antibodies and koilocytosis or the degree of CIN. Approximately 50% of patients within each grade of dysplasia had antibodies, and equal numbers of those with and without koilocytes were positive. (Table 30). One hundred and two (70.8%) of the patients with antibodies knew they had had skin warts. On the other hand, equal proportions of those who gave a past history and of those who had no known history actually had detectable antibody. (Table 31).

Positive E.I. using disrupted HPV-1 as antigen ranged from 1-19.49 whereas with disrupted HPV-2 as antigen, the highest mean E.I. was 2.89. These results are shown in Fig.18. The greater antibody response both in size and in numbers may reflect the greater exposure to antigen following HPV-1 induced lesions with a high virion content. When purified disrupted BPV was used as the coating antigen and antibodies to this assayed in 55 patients, very low absorbances (< 0.13) suggesting negligible exposure to BPV, were obtained in all patients. Nevertheless, the same patients demonstrated absorbances ranging from 0.23 to 1.21 (E.I.=0.02-7.52) against HPV-1 and from 0.24 to 0.56 (E.I.=0.08-2.61) against HPV-2. The significance of these results will be discussed.

2.2 Plasma IgG against Specific Gene Products

Although only small quantities of fusion proteins were available, a few attempts were made to see if antibodies to potential gene products could be detected using the standard indirect ELISA.

All four fusion proteins of HPV-1 and HPV-2 provided by Dr. John Doorbar were tested (i.e. HPV-1, L1, L2 and E2 and HPV-2 L2) on a small group of specimens previously tested with disrupted HPV-1 and HPV-2 antigens. While concentrations of HPV-1 and HPV-2 L2 in the range 30-50 µg/ml gave many high absorbances (range 0.26-1.23; mean 0.52 ± 0.30) and a concentration of 1µg/ml

Table 31 Influence of skin wart history on antibody response to HPV-1 and HPV-2 in patients with CIN

Skin wart history	No. of patients	No. with E.I. > 1.00 to			Total No. positives	Presence (K+) or absence (K-) of koilocytes in positive responders	
		HPV-1 only	HPV-2 only	Both HPV-1 & 2			
Past or present	196	50	5	47	102 (52.0%)	51 K+	51 K-
No known history	86	23	2	17	42 (48.8%)	17 K+	25 K-
Totals	282	73	7	64	144 (51.1%)	68 K+	76 K-

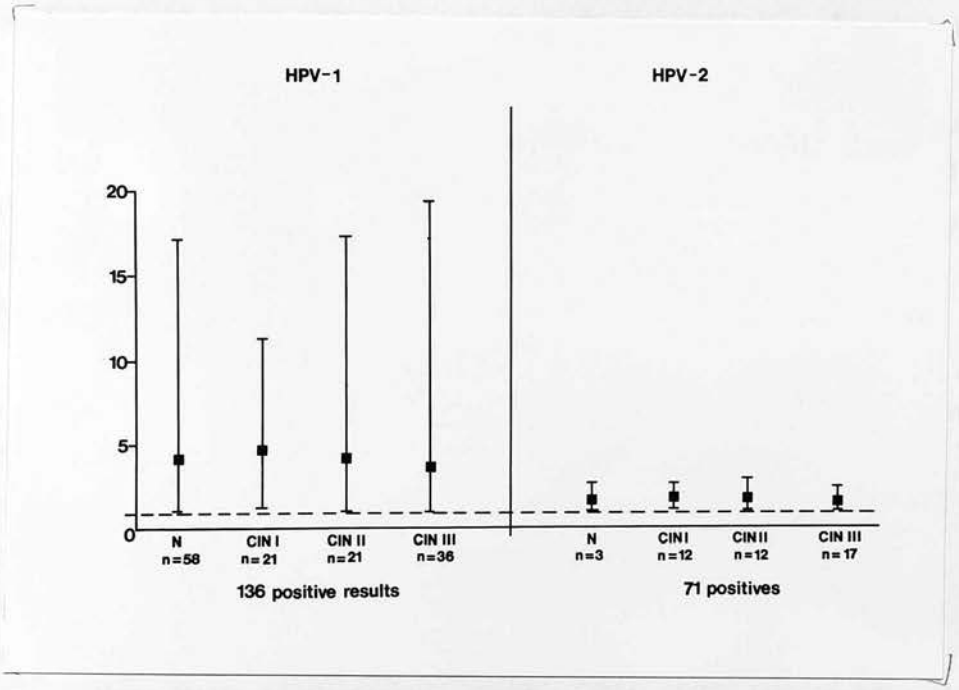


Fig 18 ELISA indices using HPV-1 and HPV-2 antigens in patients with varying degrees of CIN

gave little more than background levels (0.11 ± 0.07), concentrations of 3-5µl/ml gave a wide scatter in the range 0.10-1.10. Nevertheless, there was no correlation with the results previously obtained. When plasma from colposcopy patients, laboratory staff and patients with recalcitrant common warts were tested with all four fusion proteins at concentrations of 3-5µg/ml, wide variations in absorbance were again obtained but no pattern emerged. Frequently the absorbance with HPV-1E2 was higher than with the late gene products, but this was also found in specimens from people who would not be expected to respond to HPV antigens including a twoyear old child. It seemed likely that reactions were being detected in many people to residual E.coli protein sequences translated with the HPV fusion protein.

A similar situation was found when "crude" HPV-16E6 and HPV-18E6 preparations were used. Plasma from several people gave high absorbancies when the proteins were used at similar concentrations, but identical reactions were obtained with HPV-16E6 and HPV-18E6 suggesting that some antigen common to both preparations was being recognised and not the papilloma product itself. Again upstream E.coli sequences may be involved.

Very little of the highly purified HPV-16E6, HPV18-E6 and control protein was available for ELISA. Preliminary assays using antigen coated at 0.35µg/ml and plasma from subject 1 and 3 colposcopy patients were set up. Similar absorbancies over most wells suggested that the concentration of fusion protein was too low, allowing nonspecific attachment of plasma proteins during the 2½ hour incubation period, despite previous blocking with 3% BSA. Ten plasmas from colposcopy patients, plasma from subject 1 and from two children under 5 years of age, were tested against HPV-16E6, HPV-18E6 and then control protein coated at 2.5µl/ml. Negligible absorbancies (0.01-0.27, $\bar{x}$ =0.07± 0.06) were obtained from HPV-16E6 and HPV-18E6 although 5 colposcopy patients and one child gave

much higher absorbances with the control protein ($\bar{x}$ = 0.58 $\pm$ .20). Unfortunately, no further antigen was available to explore this system further.

2.3 Secretory IgA in Cervical Washings

The presence of secretory IgA was investigated in 24 specimens of cervical washings using an indirect ELISA with SDS.ME disrupted HPV-2 as antigen and rabbit antihuman secretory component conjugated to horseradish peroxidase as the detecting reagent for bound sIgA.

The first secretions were collected in 2ml of distilled water and diluted from 1 in 2 to 1 in 256 for the test. A prozone effect was observed in all specimens, presumably due to the presence of proteolytic enzymes in the secretion, a blocking effect of excess mucus or to sub-optimal salt concentrations in the first dilutions. Subsequent specimens were collected in 1ml of distilled water and tested at dilutions starting from 1 in 20 to circumvent this problem. Since plasma does not contain secretory IgA, background absorbances were calculated in each run by including at least 6 wells with plasma from the same patients or from laboratory staff where the appropriate plasma was not available. The cut off was taken as the mean + 3S.D. of the baseline. To detect free secretory component, washings at the starting dilution were added to uncoated wells. High absorbances in these wells suggested a direct binding of the conjugate to secretory component attached to the solid phase.

Most of the samples gave absorbances lower than the cut-off at all dilutions tested. However, 8 samples (33%) gave higher absorbances. E.I. were calculated for these samples by dividing the absorbance of the specimen by the cut-off absorbance obtained in the same assay, and these are detailed in Table 32. It can be seen that 6/8 were positive at the starting dilution with the

Table 32 Presence of secretory IgA in cervical washings in 8 patients attending the Colposcopy Clinic

Specimen No.	Histological Appearance	Starting Dilution	E.I.	Titre	E.I. at this dilution
88134	CIN I K+	32	**1.80**	> 256	**1.30**
88135	CIN I K+	32	**1.80**	> 256	**1.85**
88137	CIN III K-	32	**1.85**	64	**1.40**
88230	No biopsy	20	**1.59**	50	**1.05**
88231	CIN II K+	20	**1.10**	20	**1.10**
88232	CIN III K-	20	**3.12**	50/100	**1.95**/0.95
88233	CIN III K-	20	0.78	50	**1.24**
88234	CIN III K+	20	0.59	50/100	**2.05/1.05**

remaining two showing a prozone even at 1 in 20. Positive reactions were not confined to patients who showed evidence of koilocytosis on histological examination. Free secretory component was detected in three of the 24 specimens on initial testing, and in two others (88134 and 88135, Table 32) it was detected following storage of the specimen at 4°C during which time the specific anti-HPV-2 secretory IgA disappeared.

These preliminary results suggest that an indirect ELISA to measure specific secretory IgA in cervical washings could be developed. Standardisation of the starting dilution of specimens used would be required, preferably by estimation of the protein content of each sample.

3. Histochemical Studies

3.1 ISH with Cloned Probes

Adherence of the sections during pre-hybridisation processing was found to be one of the biggest technical difficulties encountered and was solved by a combination of slide-coating and careful control of protein digestion. The first successful ISH results were obtained using slides coated with 1% poly-L-lysine but approximately 30% of the sections tested were lost before hybridisation could be completed. The chemical silanation method of coating slides described in early 1987 by Tourtelotte and co-workers proved far superior to poly-L-lysine and, provided the slides were used within 8 weeks of activating, 100% adhesion of sections was obtained. The quick silanation method of 2% aminopropyl triethoxysilane in acetone (Burns et al., 1987) was also much more efficient than poly-L-lysine, but with the few specimens treated with this method, occasional sections were still lost.

Proteinase K was used to unmask target DNA by partial digestion of cellular proteins. A standard concentration of 0.2mg/ml was eventually chosen because higher concentrations left islands of

separated cells attached to the slide and the morphology of the section as a whole was lost. Lower concentrations occasionally yielded falsely negative results and also gave more background staining.

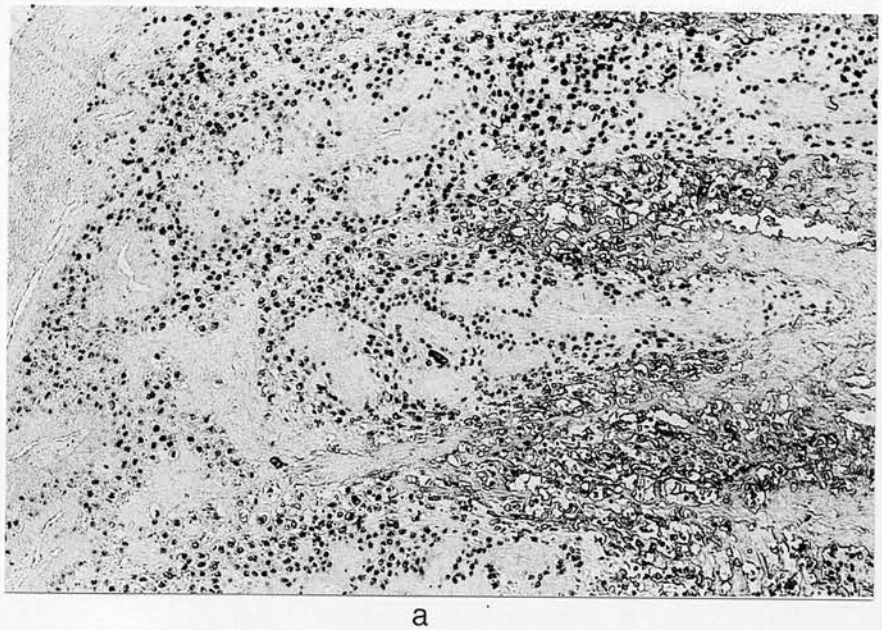
Having established a satisfactory method, (see Methods, Section 2.331) sections from seven skin lesions, paraffin embedded after fixation in formalin or Bouin's solution were examined (Table 33). While a hyperkeratotic hand wart (specimen 1) gave strong positive reactions on many occasions and was used subsequently as a positive control, HPV infection in two other skin warts (specimens 2 and 3) was detected only by the presence of HPV antigen, (vide infra) HPV-DNA, or under low stringency suggesting the presence of a different type of HPV. The extensive spread of HPV-DNA-containing cells in specimen 1 is shown in Fig. 19(a) and at higher magnification (b) the density of stain suggests very high copy number. With an HPV-16 probe (c), no positive nuclei were observed. By contrast, the distribution of positive nuclei in the vulval wart (specimen 7) was patchy (Fig. 20a) and, as noted by Chou et al., (1987) preferentially found in the crevices of the epidermis (Fig. 20b). HPV antigen in this specimen was confined to the upper layers in the crevices (vide infra).

The method was then applied to paraffin sections of ten cervical biopsies, mostly showing moderate to severe dysplasia and obvious koilocytosis when examined histologically. Three stained positively in a few nuclei with the HPV-16 probe, while one other was weakly positive on one occasion and a fifth was weakly positive with the HPV-11 probe (Table 34). HPV antigen was not detected in any of the seven tissues stained (specimens 9-13 and 17). Although all seven patients had antibodies to HPV-1 and/or HPV-2, only two had given a positive reaction in LPA (specimen 11 gave an S.I. of 2.95 to purified HPV-1 and specimen 17 gave an S.I. of 3.57 to disrupted HPV-1). There was no correlation between the detection

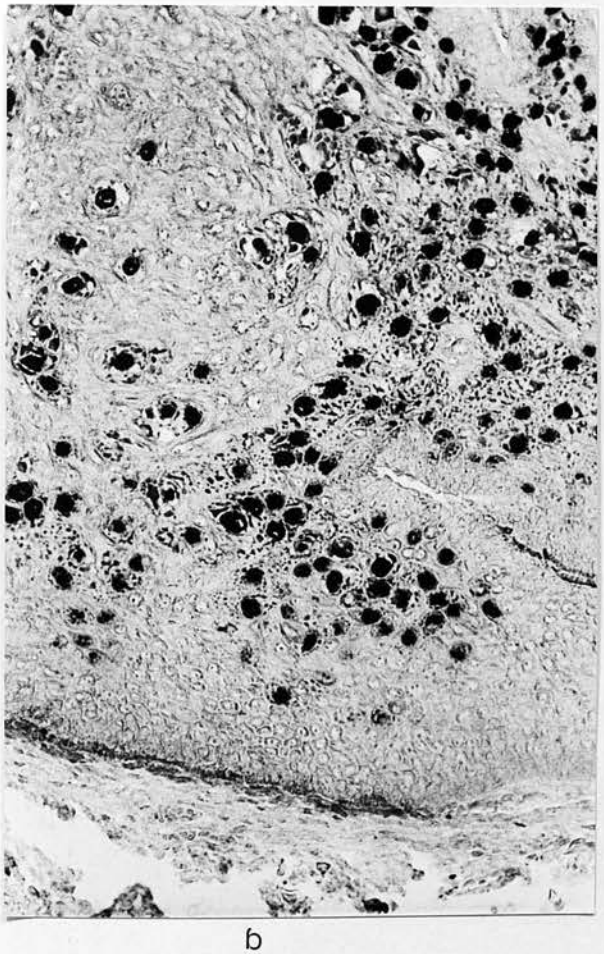
Table 33 ISH results using biotinylated cloned HPV probes on paraffin sections from 7 papillomatous skin lesions (BRL-DNA detection system)

Specimen No.	Histological Appearance	HPV antigen detected	ISH results with probes:* HPV-1	HPV-11	HPV-16	pBR322/0/λ
1	Hyperkeratotic hand wart	+++	+++	-	-	-
2	Viral wart	+	-	-	-	-
3	Simple wart	-	-*	-	-	-
4	Seborrhoeic wart	ND	-	-	-	-
5	Squamous papilloma	-	-	-	-	-
6	Rectal mucosa from edge of anal wart	ND	-	-	-	-
7	Vulval warts	+	-	+	-	-

** = results obtained with high stringency buffer (50% formamide)
* = positive result obtained with low stringency buffer (10% formamide)
+++ = frequent patches of nuclei containing HPV DNA (or antigen)
+ = infrequent patches of less well stained nuclei
- = no positive nucleic observed
ND = not done, or sections lost on processing.



a



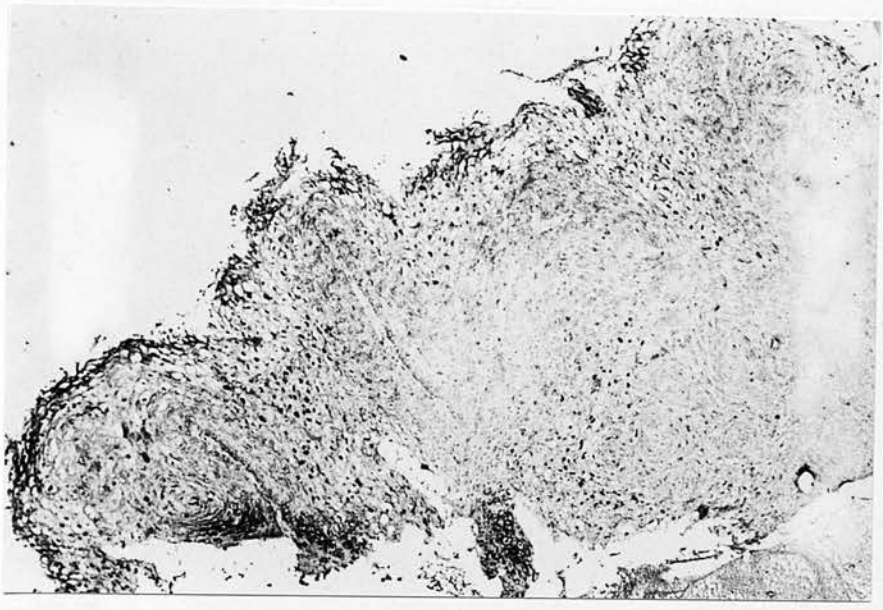
b

c

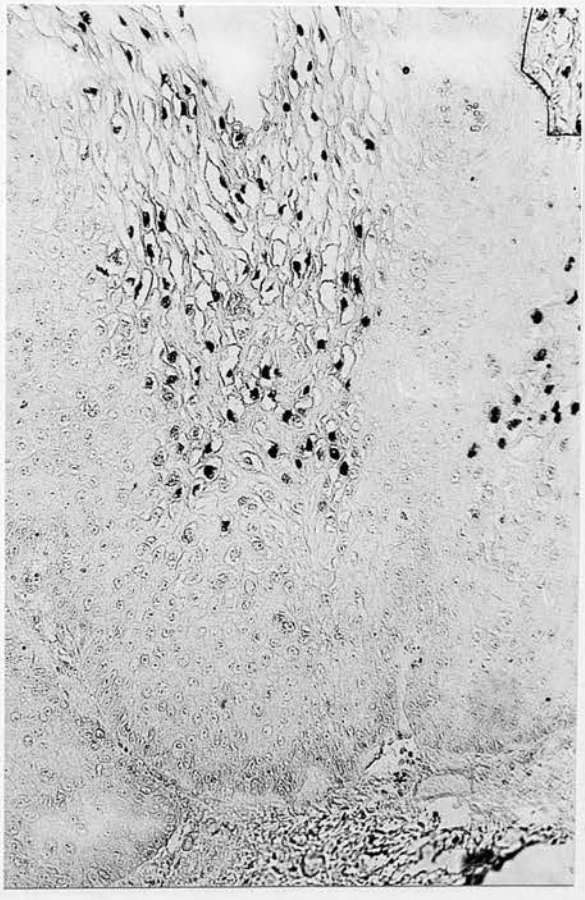
Fig 19

In situ hybridisation in skin warts : sections of a hyperkeratotic hand wart (Specimen 1) hybridised with

a) biotin labelled cloned HPV-1 DNA, showing extensive nature of infected cells x 50
b) biotin labelled cloned HPV-1 DNA showing intranuclear localisation of stain within stratum granulosum x 160
c) biotin labelled cloned HPV-16 DNA showing no positive staining (x 160)



a



b

Fig 20

In situ hybridisation in vulvar warts : sections (Specimen 7) hybridised with cloned HPV-11 DNA showing

a) patchy distribution of positive nuclei x 50

b) accumulation of positive nuclei in epidermal crevices x 125

Table 34 ISH results using biotinylated cloned HPV probes on paraffin sections from 10 cervical lesions showing dysplasia and koilocytosis (BRL-DNA detection system

Specimen No.	VRL No.	Histological Appearance	ISH results with probes: HPV-1	HPV-11	HPV-16
8	86023	CIN II K+	ND	ND	-
9	86087	CIN II K+	ND	-	-
10	86150	CIN I K+	-	ND	+
11	86197	CIN III K+	-	-	-
12	86422	CIN II K+	-	-	+
13	86471	CIN III	-	-	(+)
14	86504	CIN III K+	-	-	-
15	86508	CIN III K+	-	-	-
16	86509	CIN III K+	-	(+)	-
17	86695	CIN II K+	-	-	+

+ = infrequent patches of less well stained nuclei

(+) = HPV-containing nuclei only found with careful searching

- = no positive nuclei observed

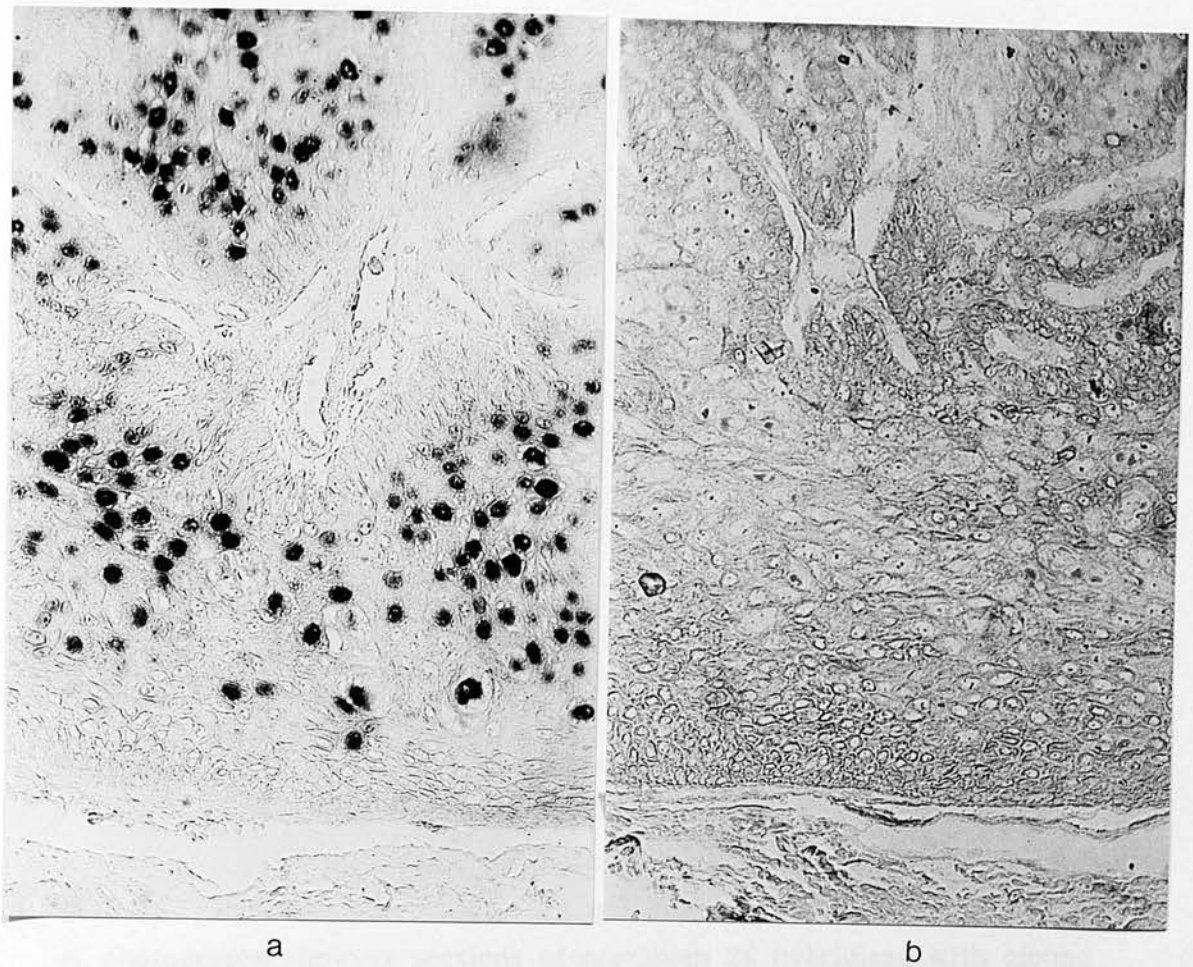
ND = Not done, or sections lost on processing

of HPV-11 or HPV-16 sequences in tissue sections and the LPA result with a number of different antigens. The sensitivity of biotin-labelling is generally considered to be lower than that of radioactively labelled probes (Syrjanen et al., 1987b) and it was felt that the method used here was not sensitive enough to allow adequate comparison with the results of immunological assays.

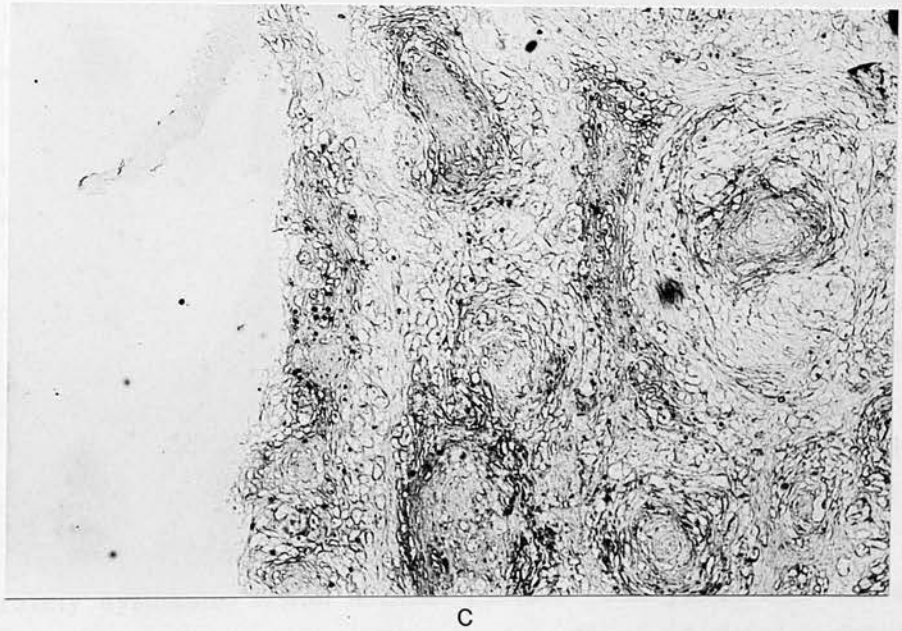
3.2 ISH with Synthetic Oligonucleotide Probes with Streptavidin-biotin Amplification

The three synthetic probes from the E6 region of HPV-1a, HPV-6b and HPV-16, prepared as described in section 1.32 and Fig. 9 in the Methods were applied to paraffin sections of skin and genital wart tissues mounted on silanated slides to assess their usefulness in in situ hybridisation reactions. A shorter prehybridisation processing was required because of the small size of the probe and, as suggested by Lin et al. (1987) the 16-18 hour hybridisation stage was replaced by a 2 hour incubation at 42°C. The results are presented in Table 35. Two skin warts which stained strongly with the HPV-1E6 probe (Fig. 21) acted as positive controls for future experiments. Specimen 1 had already been easily characterised with the cloned HPV-1 probe, while specimen 18, which had been cross-cut and was mounted on untreated slides, tended to disintegrate to small positive fragments with the cloned probe. With the oligoprobe and shorter processing, disintegration and loss of section only happened occasionally. HPV antigen was detected in both these specimens and also in two of three additional skin warts. These were both keratotic forearm warts only one of which stained positively with the HPV-1E6 probe. This reaction although strong, was not found on subsequent testing, suggesting perhaps that only a small focus of infection had occurred within this keratotic nodule which had required histological confirmation of its warty nature.

Of four genital warts, three hybridised with the HPV-6bE6 probe (specimens 7, 22 and 23) and one with HPV-16E6 (specimen 25),



a b



c

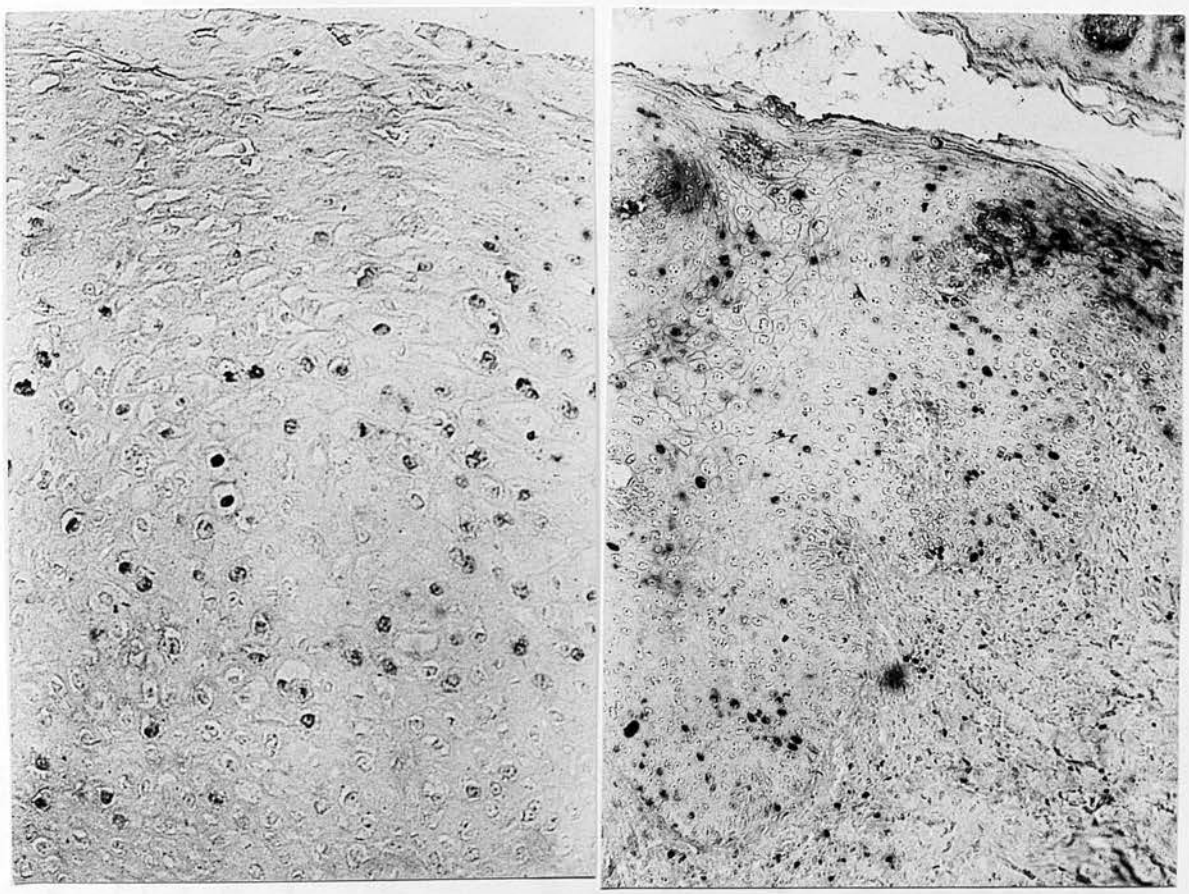
Fig 21

In situ hybridisation in skin warts : sections of hyperkeratotic hand wart (Specimen 1) hybridised with
a) biotinylated HPV-1 E6 oligoprobe x 160
b) biotinylated HPV-16 E6 oligoprobe x 160
and of a simple plantar wart (Specimen 18) hybridised with
c) biotinylated HPV-1 E6 oligoprobe x 50

while a specimen of anal tissue with no histological evidence of papilloma infection was negative (specimen 24). Examples of the hybridisation reaction in the vulvar wart and an anal wart are shown in Fig. 22. Interestingly, specimen 7 had previously been quite strongly positive with the HPV-11 cloned probe (see Fig 20), while the two anal warts from the same patient (specimens 22 and 23) had been negative and weakly positive respectively with the HPV-11 probe. These results suggest that some cross-hybridisation between HPV-6b and HPV-11 had occurred. One sample of vaginal warts (specimen 25) from a renal transplant patient, was found to harbour HPV-16 sequences detectable with both HPV-16E6 oligoprobe and the cloned probe. The patient had had a vulvar carcinoma one year earlier in which HPV-16 was detected by Southern blot (Rudlinger et al., 1986; see also specimen 27, Table 35). An anal wart taken two years after the vaginal wart from the same patient hybridised with both HPV-6bE6 and HPV-16E6 oligoprobes (specimen 26). A collage of photographs showing sections of specimen 26 hybridised with cloned HPV-16, HPV-16E6, HPV-1E6 and no probe is shown in Fig. 23. It can be seen that the distribution of positive cells is quite different in the HPV-16E6 and the HPV-16 stained sections, but this may reflect cutting at different times thus providing sections from different parts of the block.

In a further group of five anogenital lesions, four were positive with either HPV-6bE6, cloned HPV-11 or both, results which largely agreed with those obtained using cloned HPV-6b and HPV-11 probes individually.

Thirteen specimens of cervical lesions were examined using HPV oligonucleotide probes (Table 36), including one (specimen 41) from the same patient as specimens 25-27 in Table 35. This moderately dysplastic lesion without obvious koilocytosis also contained sequences which hybridised to HPV-16E6. Three of the remaining twelve lesions also hybridised with HPV-16E6, two of which were



a b

Fig 22

In situ hybridisation in genital warts hybridised with biotinylated HPV-6b E6 oligoprobe :

a) Specimen 7, showing scattered well-stained nuclei x 160

b) Specimen 22, showing scattered and variably stained nuclei x 125

Table 35 ISH results using biotinylated synthetic oligonucleotide probes on paraffin sections from 17 skin lesions (BRL-DNA detection system)

Specimen No.	Histological Appearance	HPV antigen detected	ISH results with Synthetic oligoprobes HPV-1E6	HPV-6bE6	HPV-16E6	Cloned probes HPV-1	HPV-11	HPV-16	6/11/16/18%
1	Hyperkeratotic hand wart	+++	+++	-	-	+++	-	-	
18	Single plantar wart	++	++	-	-	++	-	-	
19	Cheek wart	-	-	-	-	-	-	-	
20	Keratotic forearm wart from same patient as no 19	+	-	-	-	-	-	-	
21	Keratotic forearm wart	+	+/-*	-	-	-	-	-	
7	Vulvar warts, K+	+	-	+	-	-	++	-	
22	Perianal warts, K+ severe dysplasia	ND	-	+	-	-	-	-	
23	Anal wart, same patient as no. 21, 1yr later	-	-	+	-	-	(+)	-	
24	Anal tissue, not wart	ND	ND	-	-	ND	ND	ND	
25	Vaginal warts, K+	-	-	-	+	-	-	-	
26	Anal wart, same patient as no. 25 2yrs later	ND	-	+	+	-	-	+	
27	Vulvar carcinoma, same patient as no.25 1yr earlier	ND	-	ND	(+)	ND	ND	-	
28	Perianal wart from homosexual male. Mild dysplasia	NA	NA	-	NA	NA	(+)	+	+ - -
29	Perianal wart from homosexual male	+	NA	+	NA	NA	+	+	+ - -
30	Perineal wart from female	NA	NA	+	NA	NA	-	+	+ - -
31	Perianal wart from homosexual male	NA	NA	-	NA	NA	+	-	+ - -
32	Perianal wart from heterosexual male	NA	NA	-	NA	NA	-	-	- - -

* +/- = clear nuclear staining found on one occasion but not on repeat testing.

% = In situ hybridisation with single biotinylated cloned probes carried out by Mr Paul Bishop, Department of Genito-urinary Medicine. ND = not done; NA = no slide available

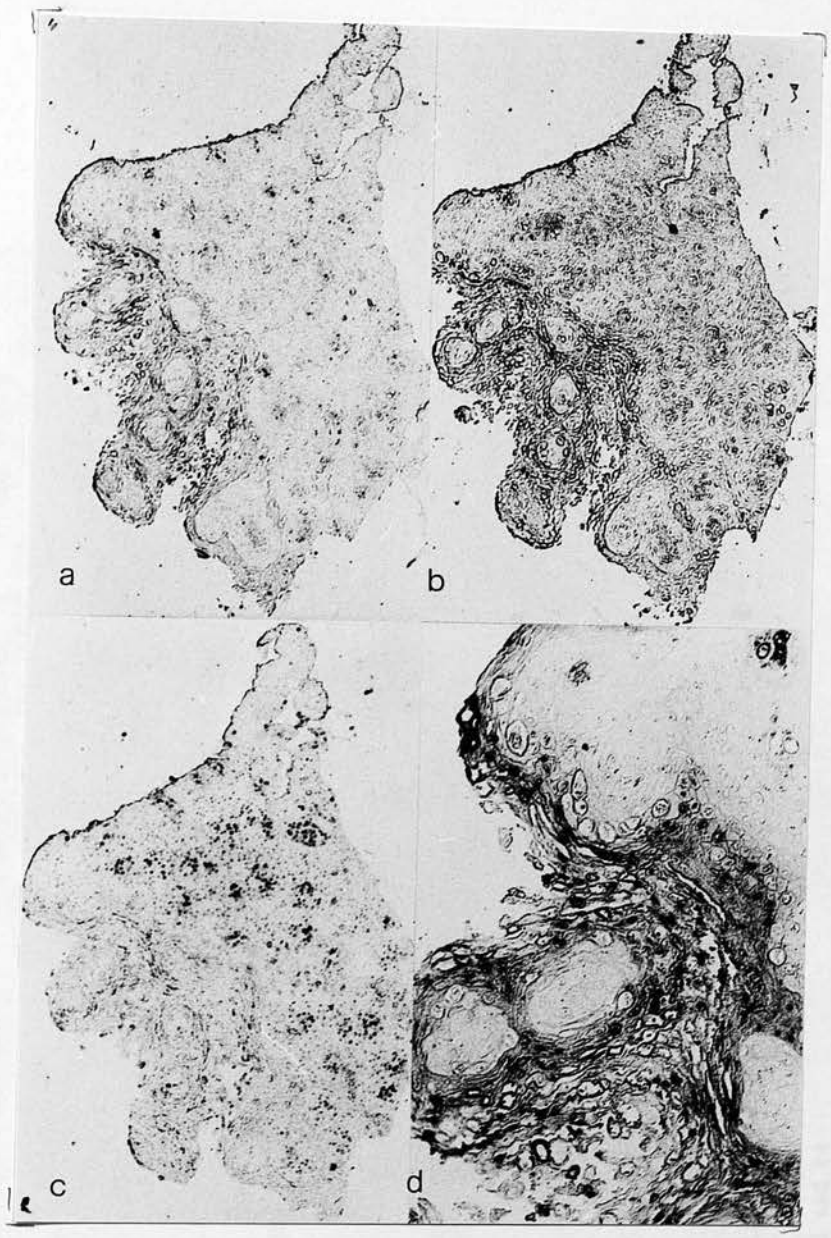


Fig 23

In situ hybridisation on sections of an anal wart (Specimen 26) showing

a) no staining, without probe x 50
b) no staining, with HPV-1 E6 oligoprobe x 50
c) positive nuclei in small patches of epithelium, in parabasal layers with HPV-16 E6 oligoprobe x 50
d) positive nuclei in one area only, of surface epithelium, with cloned HPV-16 probe x 160

Table 36 ISH results using biotinylated synthetic oligonucleotide probes on paraffin sections from 13 cervical lesions (BRL-DNA detection system)

Specimen No.	VRL No.	Histological Appearance	LPA response (S.I.)	HPV antigen in sections	ISH results with: Synthetic oligoprobes HPV-1E6	HPV-66E6	HPV-16E6	Cloned probes HPV-1	HPV-11	HPV-16
33	88131	No dysplasia K-	Neg	-	-	-	-	ND	ND	ND
34	87471	No dysplasia K-	Neg*	-	-	-	-	ND	ND	ND
35	87538	CIN I, K+	Neg	-	-	-	-	ND	ND	ND
36	86150	CIN I, K+	Neg	-	-	+	-	-	-	(+)
37	87432	CIN II, K-	**2.3/16E6**	ND	-	-	-	ND	ND	ND
9	86087	CIN II, K+	Neg	-	-	+x1	+x1	-	-	-
12	86422	CIN II, K+	Neg	-	-	(+)	+	-	-	+
17	86695	CIN II, K+	**3.6/disrupted CsHl**	-	-	-	+x2	-	-	+x2
38	88056	CIN II, K+	Neg	-	-	-	-	ND	ND	ND
39	88057	CIN II, K+	**2.1/18E6**	-	-	-	-	ND	ND	ND
11	86197	CIN III, K+	Neg	-	-	-	-	-	-	-
40	88139	CIN III, K+	Neg	-	-	-	-	ND	ND	ND
41	.	CIN II*	.	-	-	-	+	ND	-	+

* = repeat specimen 6 months later gave S.I. of 3.2 to HPV-18E6, 3.8 to HPV-16E4 and 2.6 to E4-Beta galactosidase control protein. See patient 12 Figs R3 and R4

x1 = positive results to HPV-6bE6 and HPV-16E6 were obtained or sections taken from different paraffin blocks of biopsies taken at the same time

x2 = one block was positive for both HPV-16E6 and cloned HPV-16 while another two blocks were negative with both

* = same patient as specimens 25-27 see Table 35, specimen taken at same time as specimen 25.

detectable with the cloned HPV-16 also. In addition HPV-6bE6 sequences were also found in two (specimens 9 and 12). These three specimens were all from CIN lesions of moderate severity showing koilocytosis. Only one, however, came from a patient whose PBM had responded in LPA to a disrupted PV antigen (speciment 17). Despite positive S.I. to HPV-16E6 and HPV-18E6 in two patients (84732 and 88057), HPV-16/18 sequences were not detected in the biopsies. A further specimen from a mild lesion with koilocytosis (specimen 36) hybridised with the HPV-6bE6 probe although a weak reaction with the HPV-16 cloned probe was also noted. PV antigen was not detected in any specimen. The positive reactions with HPV-16E6 in specimens 41 and specimen 12 are shown in Fig. 24.

No correlation could be found between detection of HPV sequences and the degree of dysplasia, nor between the presence of HPV-DNA in cervical lesions and a responsiveness in the peripheral blood of patients to PV antigens of various types. The detection of HPV sequences in 5/13 (38.5%) of lesions with CIN is low and suggests that the sensitivity of the hybridisation system is still too low.

3.3 ISH using Synthetic Probes with an Immunogold Detection System

The recent introduction of a monoclonal anti-biotin antibody (Dako Ltd.) coupled with the use of the immunogold-silver staining (IGSS) technique developed by Holgate et al., (1983) provided an alternative detection system to the biotin-streptavidin-biotinylated alkaline phosphatase. A two stage indirect technique using rabbit anti-mouse immunoglobulins absorbed to colloidal gold was used and bound gold particles were enhanced by the precipitation of silver onto the gold to give a black reaction product (Fig.25).

Two cutaneous warts (Specimens 1 and 18) gave positive reactions with this method with HPV-1E6 oligoprobe and two genital warts (Specimens 7 and 23) were positive with HPV-6bE6. Of eight

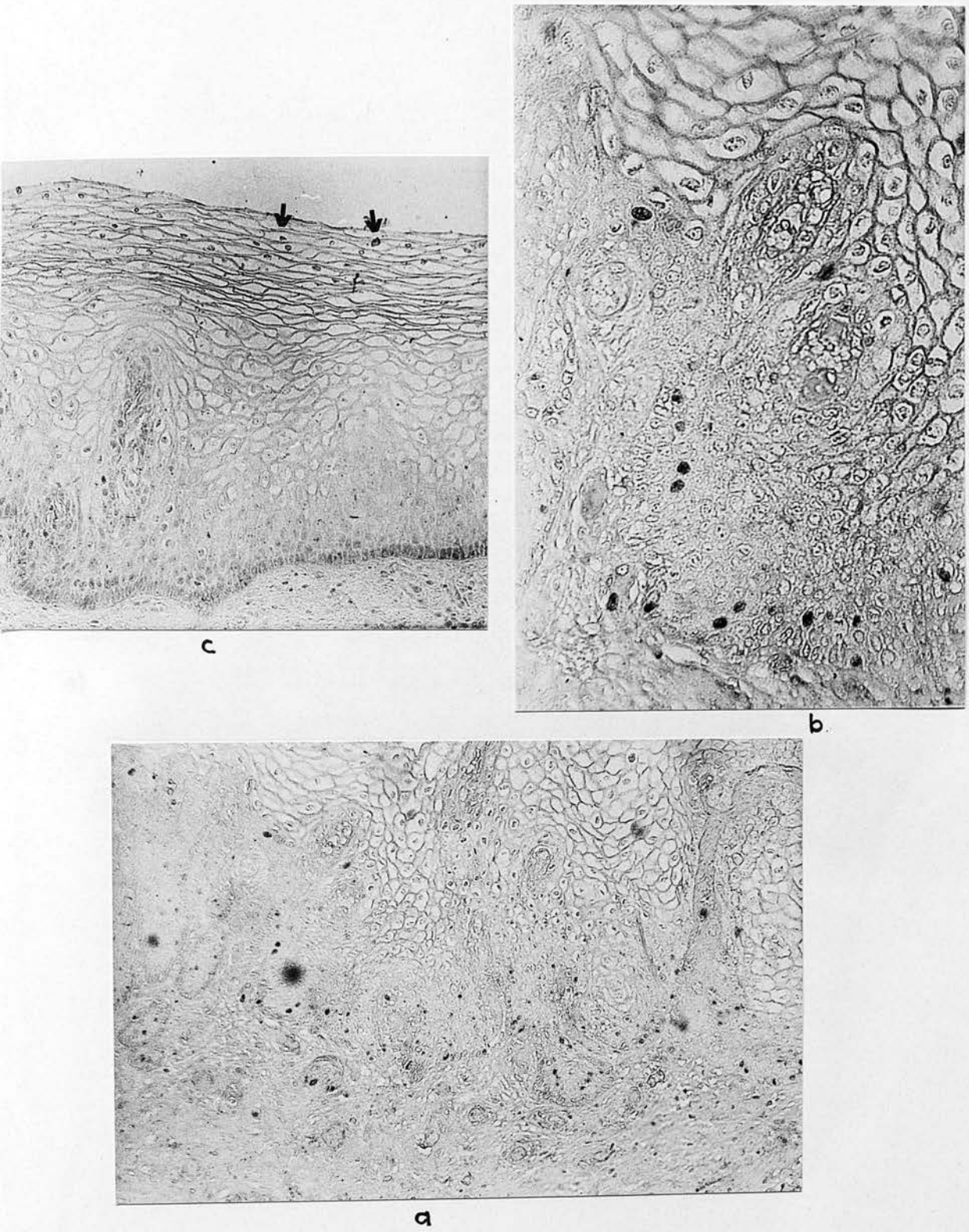


Fig 24

In situ hybridisation on sections of cervical lesions using synthetic oligonucleotide probes

a) Specimen 12 with HPV-16 E6 probe x 125. Showing densely stained cells in lower layers and a focus of positive cells in the stratum spinosum.

b) Specimen 12 with HPV-16 E6 probe x 320. Showing isolated positive cells at higher magnification.

c) Specimen 41 with HPV-16 E6 probe x 125. Showing some non-specific basal layer staining and a few positive surface cells (arrow).

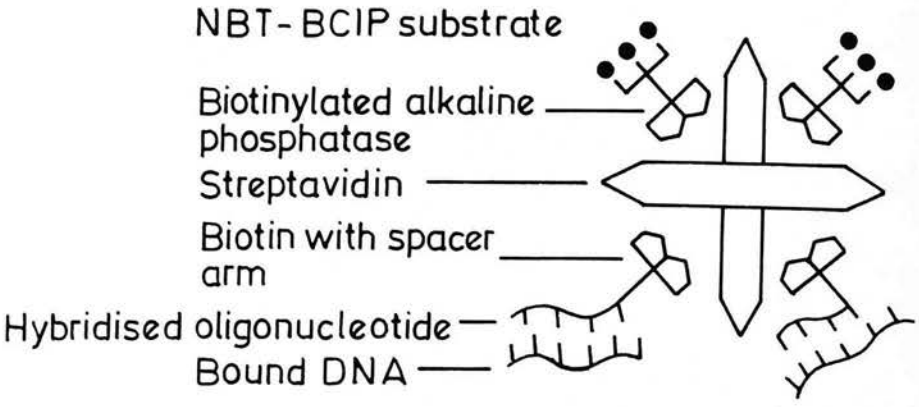
Fig 25

Diagrammatic representation of ISH-detection systems used

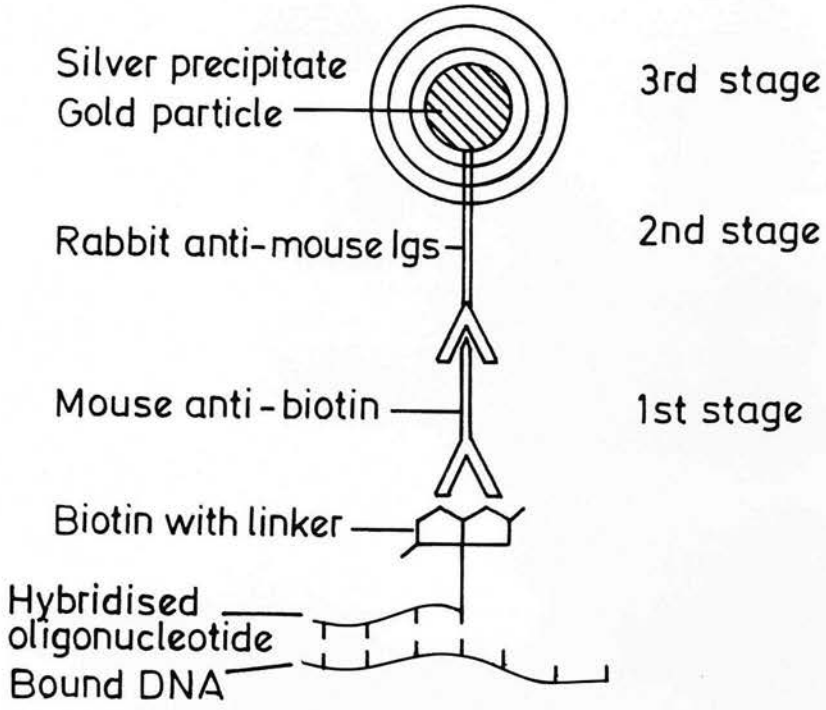
a) Method 1:DNA-biotin/streptavidin/biotinylated alkaline phosphatase - NBT/BCIP

b) Method 2:DNA-biotin/anti-biotin/gold-conjugated anti-mouse/silver enchancement.

Biotin - streptavidin - biotinylated alkaline phosphatase detection system



Biotin - antibiotin - IGSS detection system



cervical lesions, four gave positive results with HPV16E6 (specimens 17, 34, 38 and 39, Table 36) only one of which had been recorded with streptavidin-biotinylated alkaline phosphatase (specimen 17). All other conditions of the assay were the same. While inadequate reagents were available to test the anti-biotin and IGSS system further, it was felt that in this small series, it showed a greater sensitivity and was quicker to perform since the silver enhancement stage took approximately 10 minutes compared with 1-2 hours for the NBT/BCIP substrate to develop. Great care had to be taken, however, to stop the reaction before auto-catalysis of the silver occurred leading to the deposition of a non-specific precipitate. Fig. 26 shows specimen 39 in which two areas of the biopsy stained well with HPV-16E6. Their distribution can be seen in (a) while the dense black silver precipitate is more readily visible at higher magnifications ((b) and (c)). HPV-6bE6 oligoprobe gave minimal staining in one tiny corner of the biopsy (d) and at higher magnification, this is apparent as a pale grey deposit. This may represent a small focus of infection with a second virus, HPV-6b, or it may represent the level of non-specific "grey" background expected with this developing reagent. Fig. 27 shows specimen 34, which, like specimen 39 was negative with streptavidin-biotinylated alkaline phosphatase detection. A long stretch of epithelial cells in the granular and spinous layers contained positive nuclei of varying intensities. No such reactions were observed with the HPV-6bE6 probe.

3.4 ISH on Cryostat Sections

In the hope that double staining techniques could be developed linking the detection of HPV-DNA with other cell markers such as the presence or absence of Langerhans cells, cryostat sections from 26 cervical biopsies and two skin warts were processed using different fixative protocols, different protein digestion methods and

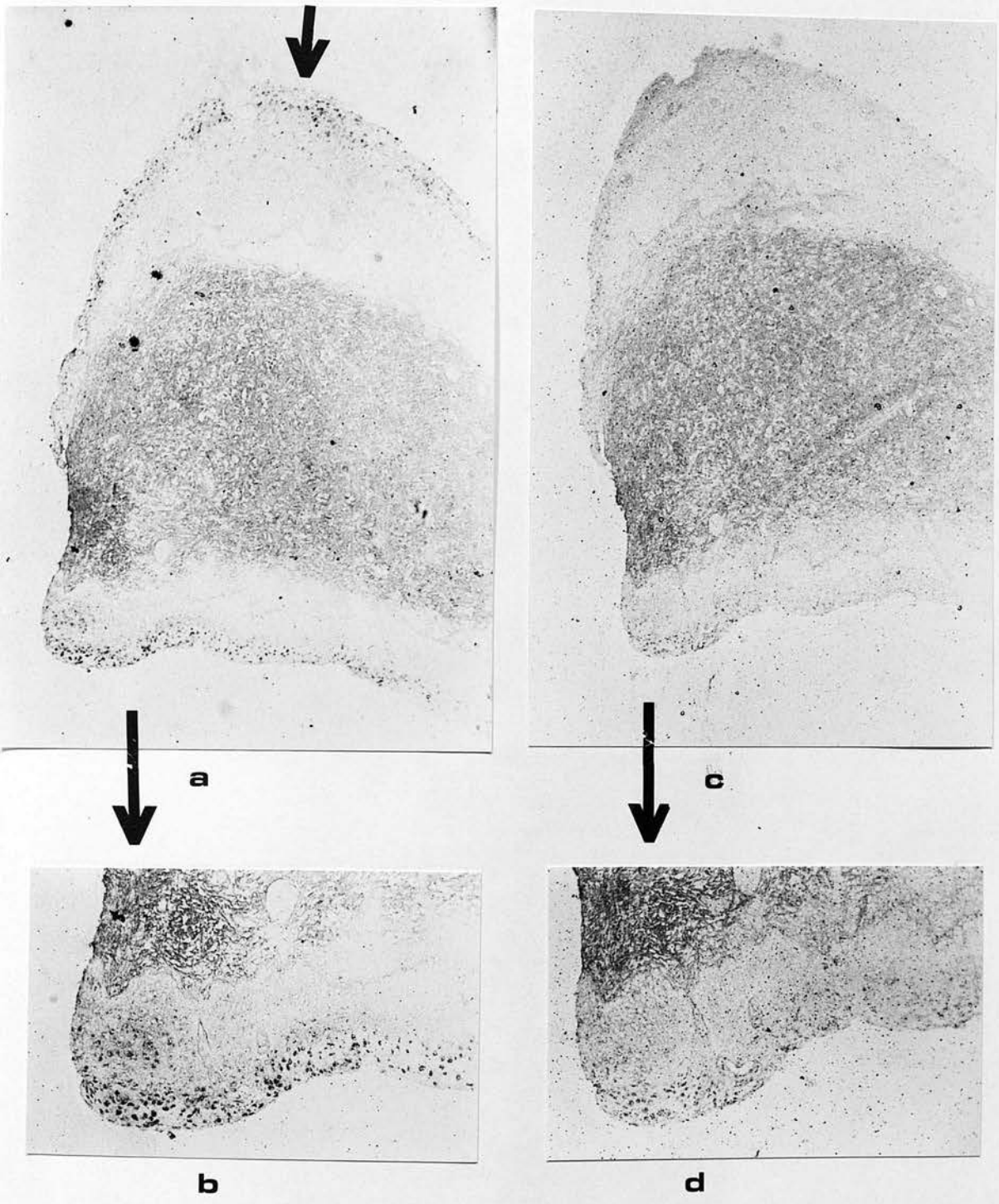


Fig 26

ISH of cervical lesions (CIN II, k+, specimen 39) hybridised with HPV-16 E6 (a, and b) and HPV-6b E6 (c and d) and detected with anti-biotin - IGSS.

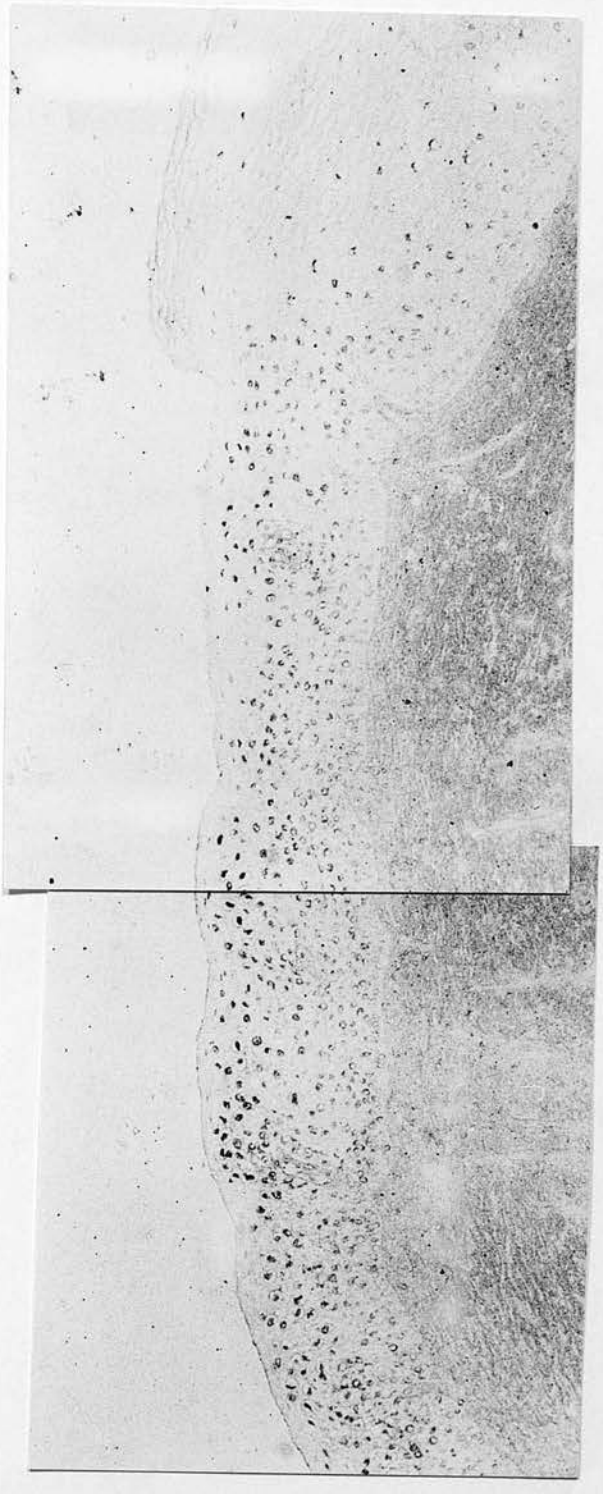


Fig 27

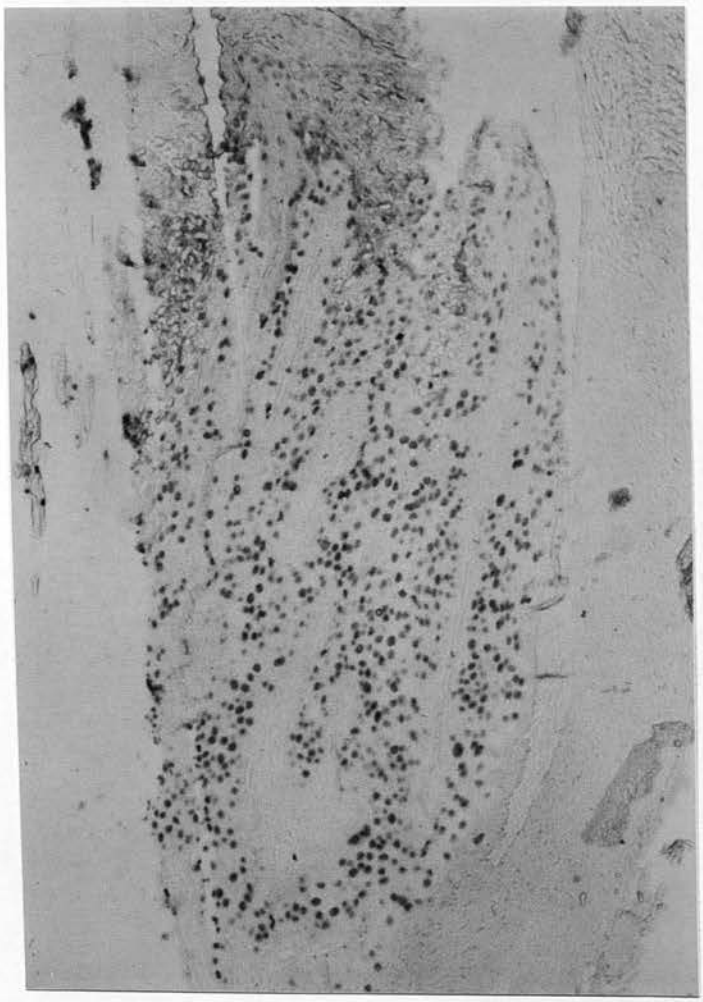
ISH of cervical lesion (with no dysplasia or koilocytosis recorded; specimen 34) hybridised with HPV-16 E6 oligoprobe and detected with anti-biotin and IGSS x 125

Fig 28

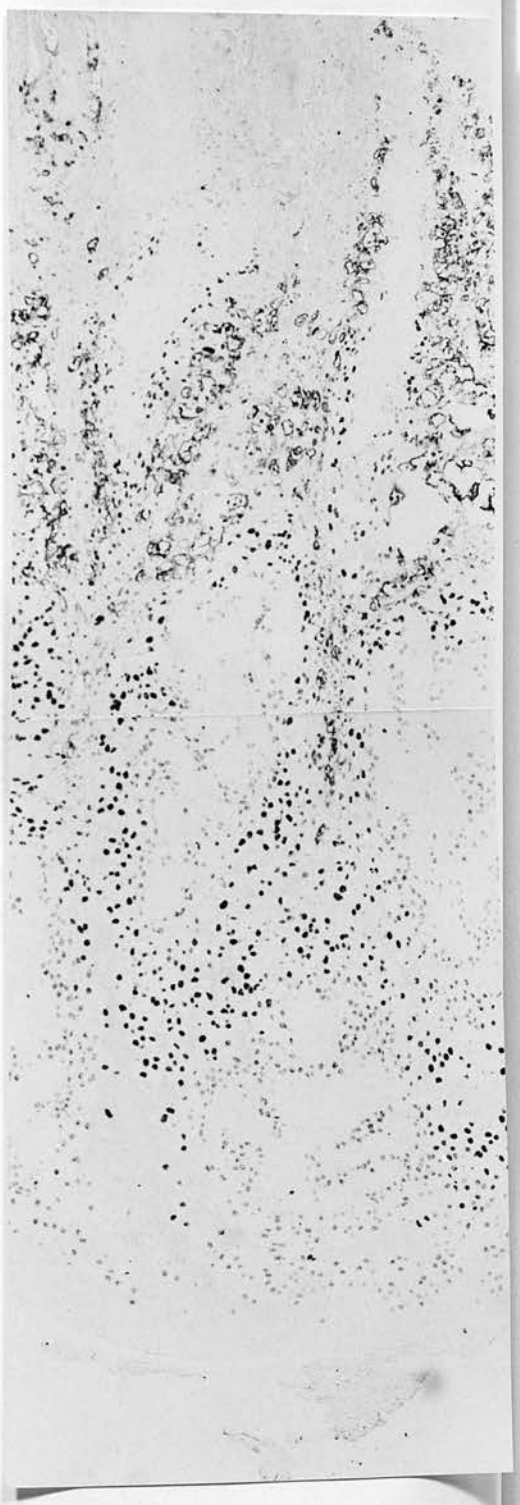
Double staining reactions for HPV-antigen (brown) and HPV-1 DNA (blue) using streptavidin - biotinylated alkaline phospatase and NBT/BCIP for detection.

a) Specimen 1 x 16

b) Collage from basement membrane to stratum corneum x 50. (DNA shows as pale nuclei and antigen/antigen + DNA nuclei stain densely).



a



b

different slide coatings. Acetone fixation, while useful for antigen preservation, was insufficient to retain cellular integrity during subsequent processing for hybridisation. Carnoy's fixative (3 parts ethanol : 1 part acetic acid, freshly made) is considered to give the best hybridisation efficiency (Haase et al., 1984) but strong background staining was frequently observed with the biotinylated probes and amplified streptavidin detection system. Preservation of sections was more adequately achieved by fixing with 4% paraformaldehyde in vanadyl sulphate (Lewis et al., 1987) and non-specific staining was reduced using their recommended detergent digestion rather than enzymic digestion. However, 4% paraformaldehyde in vanadyl sulphate proved unsuitable as a fixative for Langerhans cell markers. Further attempts to perform ISH on cryostat sections were therefore abandoned.

3.5 Double Staining

It was also of interest to link the detection of HPV-DNA with PV-specific antigens and it was originally hoped that antisera to individual gene products as well as antiserum to the group specific capsid antigen might be available. The technique was developed using rabbit polyclonal antiserum to disrupted BPV particles and biotinylated probes with streptavidin amplification as described in the Methods sections 2.34 and 2.35. Nuclei containing antigen stained brown and those containing HPV DNA stained bluish, while those containing both viral capsid antigen and nucleic acid stained a dense brownish-purple. This is shown in Fig. 28 in specimen 1, a typical HPV-1 containing wart. The brown nuclei presumably contain large amounts of PV particles whose protein coats are undigested by the pre-hybridisation conditions and into which the probe has been unable to penetrate. The production of viral capsid proteins in the upper layers of the epidermis with nucleic acid replication down to the parabasal layers is readily seen.

Double staining was also achieved with the antibiotin/IGSS detection system and in Fig. 29a the grey DNA-containing nuclei contrast with brown antigen-containing nuclei again in specimen 1. In genital warts such as specimen 7, far less antigen is detected although DNA containing cells in the lower layers are obvious (Fig. 29b). Two cervical lesions which had previously been shown to contain HPV-16 by at least one method, but which did not contain antigen when tested separately, were stained for nucleic acid and antigen in the same section. They showed scattered grey nuclei of varying intensity containing HPV16 DNA, but only non-specific brown stain distributed cytoplasmically in superficial cells, confirming the result obtained with separate staining for nucleic acid and capsid antigen.

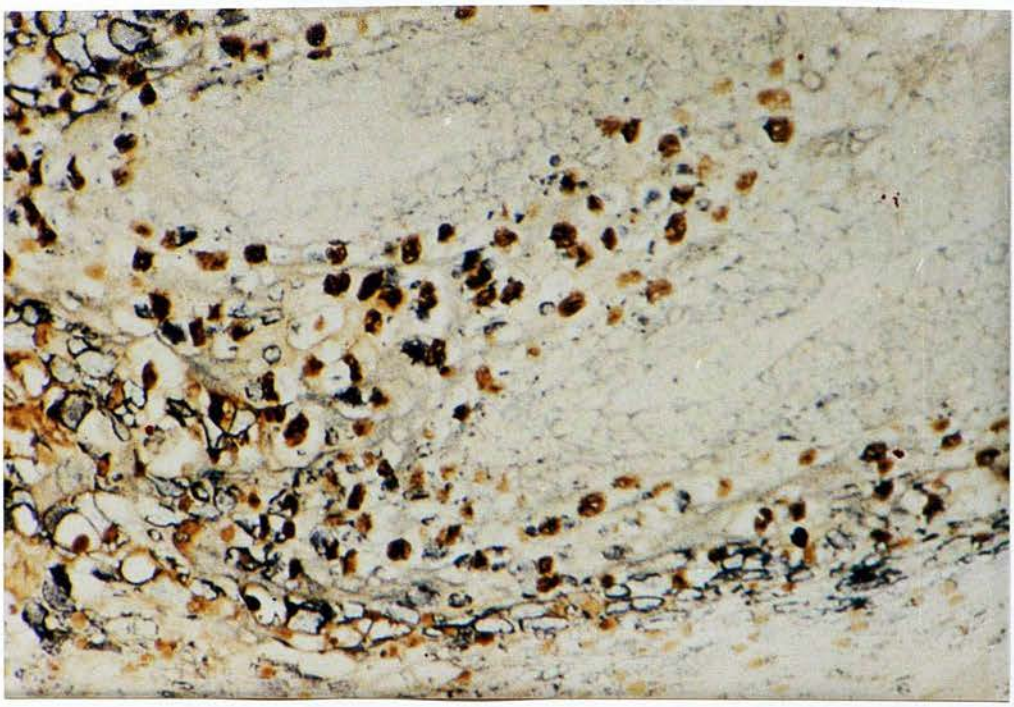
4. Mice

4.1 Subcutaneous Implantation of Cervical Biopsies

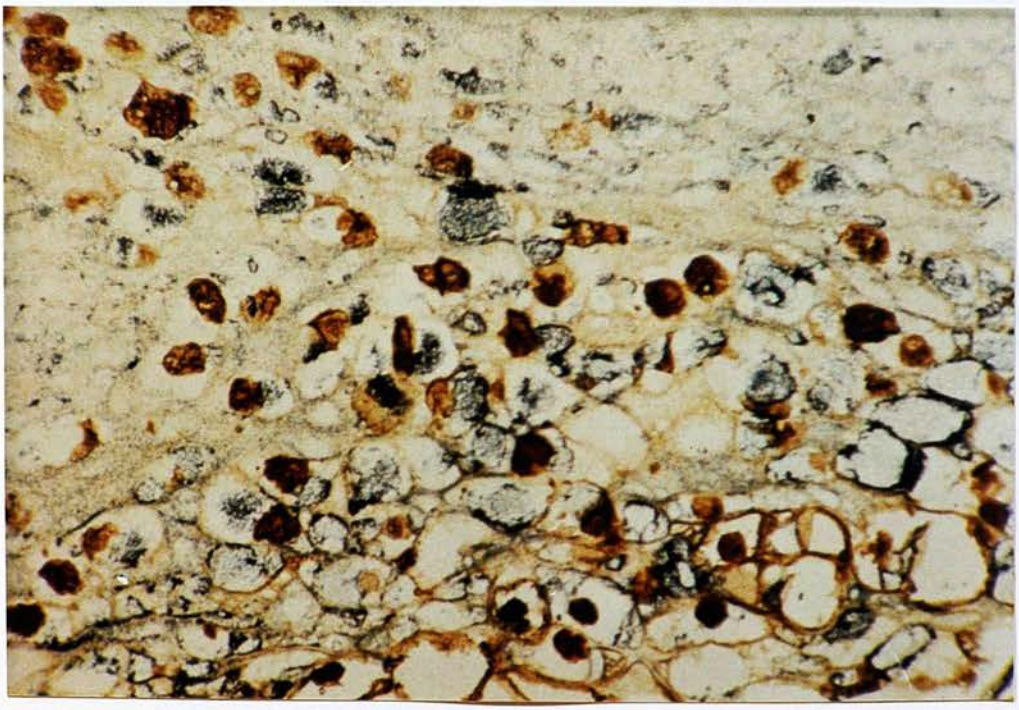
The 35 mice which were inoculated subcutaneously with small fragments of cervical biopsy tissue all survived well (26-265 days, $x = 96 \pm 45$ days) with 29 (76%) surviving more than 2 months. Fourteen of the implants could not be seen macroscopically at any stage after implantation, while a further 12 were visible for the first few weeks but decreased in size as the tissue was broken down. In the remaining 12 (6 female and 6 male), the implants were visible, without growing any larger, throughout the life of the animal (39-125 days), $x = 83 \pm 38$ days). Histological examination of five of the implants showed no tumours in any although granulation tissue resembling a fibroma was observed in one (original biopsy showed no dysplasia or koilocytes histologically). A lesion with two small nodules and derived from a CIN I K + biopsy which remained *in situ* for 102 days was obtained. Histologically it showed two abortive

Fig 29

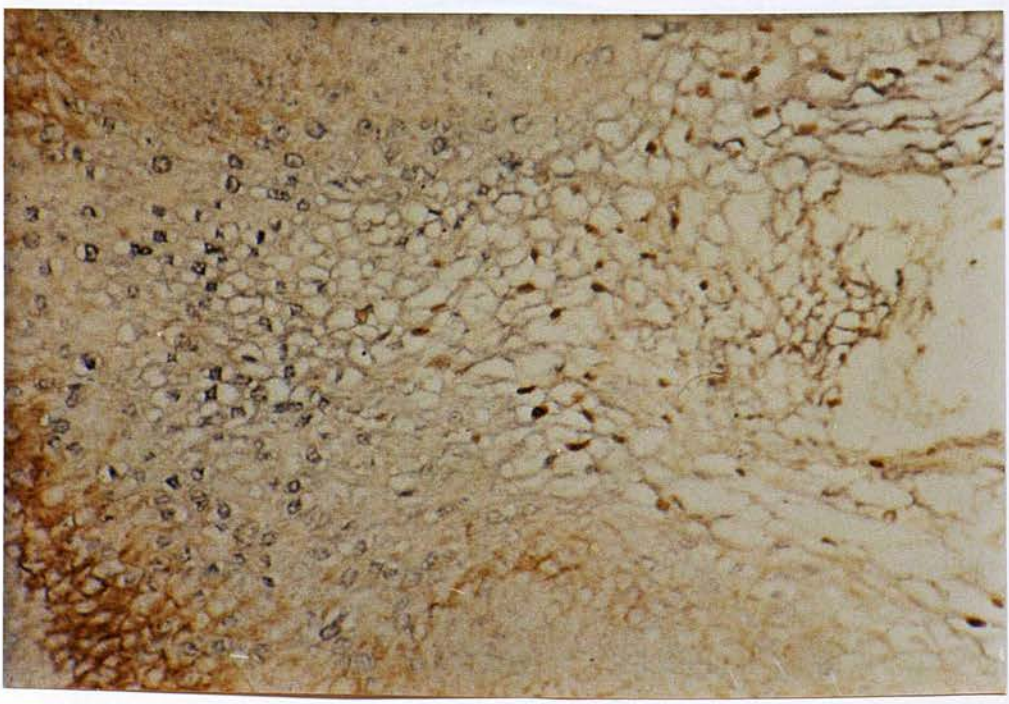
Double staining reactions for HPV antigen (brown) and HPV DNA (grey) using anti-biotin - IGSS detection of oligoprobes
a) Specimen 1
b) Specimen 1
c) Specimen 7, vulvar warts showing scant antigen.



a



b



c

foci containing stratified squamous epithelium with sloughed vacuolated and sometimes degenerate squamous looking cells, surrounded by fibrosis. One implant which remained visible for 119 days was passaged through two further mice for a total of 244 days without increase in size. Subsequent passage was unsuccessful and no biopsy remained for histology.

4.2 Implantation of HPV-infected Tissue under the Renal Capsule

Four cervical biopsies were implanted under the renal capsule of female nude mice, but no trace of them was found when the animals were killed 43, 93, 107 and 186 days later.

Human foreskin which had been suspended in mouse grown HPV-11 was implanted under the renal capsule of 8 nude mice. Of the 7 which survived, four produced no lesions during the 3-6 months that they survived thereafter. The remaining three all produced hard white cyst-like lesions, readily visible within 5-6 weeks of implantation (Fig. 30). All three were examined histologically. One appeared to be a dermoid cyst possibly of murine origin, while the other two showed thickened, papillomatous epidermis. They both appeared to be cystic keratinising neoplasms derived from human squamous epithelium, with keratin debris being sloughed into the centre of the cyst. Koilocytic cells were present (Fig 31). In addition, EM examination of negatively stained suspensions from both cysts revealed occasional viral particles in each grid square examined (Fig. 32). Further foreskin fragments have being suspended in the clarified supernate from these cysts and implanted under the renal capsule of further nude mice.

Fig 30

Visible cyst on nude mouse 5 weeks after implantation of human foreskin infected with HPV-11 under the renal capsule.

Fig 31

Section of papillomatous cyst derived from HPV-11 infected human foreskin implanted under the renal capsule of a nude mouse (stained H + E).

Fig 32

Negatively stained HPV particles from papillomatous cyst from nude mouse
Magnification x 70,000
Inset magnification x 130,000

DISCUSSION

DISCUSSION

DISCUSSION

Introduction

The studies reported here confirm that a systemic response to epithelial HPV infection can occur, but they demonstrate that the response is weak, irregular and type-specific. Discussion of the results is divided into five sections. The first section covers the use of lymphoproliferation assays (LPA) as a measure of the cell immune response to HPV and is followed by a discussion of possible pathways of development of this response. The value of tests of systemic antibody responses in HPV infections is discussed in Section 3, with the suggestion that further investigation of local antibody production would be valuable. In Section 4, technical problems associated with *in situ* hybridisation methods are outlined, the production and optimum length of synthetically produced probes and labelling of them are discussed. The different techniques used in this study are compared, and the value of detecting HPV nucleic acid in association with other markers of infection or of immunological reaction to HPV is explored. The continuing need for an adequate *in vitro* replication system for HPV is discussed in Section 5.

The final section of this Discussion covers the multi-factorial process of tumour development from initiation through promotion in the presence of different risk factors to overt progression.

1. Lymphocyte Proliferation Assays as a Measure of Systemic CMI to HPV

The use of LPA as an in vitro measure of CMI to different viruses is well documented and was first used in the 1970's to study the cellular response to skin viruses (Lee and Eisinger, 1976 to HPV; Wilton et al. 1972 to HSV) and in this laboratory the technique has been used to study exposure and response to CMV in patients with cervical dysplasia (Neill, 1984; Neill and Norval, 1985).

A valid assay of CMI must be sensitive enough to be assuredly positive for a high proportion of known infected individuals. In Lee and Eisinger's work with purified extracts from pooled warts, most patients with skin warts of short duration or with previous infections did respond although the mean S.I. was low. Similarly low blastogenic responses have been found with other viruses where persistence is common, e.g., Hepatitis B surface antigen (Tong et al., 1975) and CMV (Neill & Norval, 1985), and indeed patients with warts of more than one year's duration responded very poorly with a mean S.I. of only 1.4 in Lee and Eisinger's study. In the current investigation, 5/10 (50%) of a group of patients with recalcitrant warts of more than one year's duration responded to a glycine extract of purified HPV-2, purified HPV-2 virions or both (Table 10) with S.I. ranging from 2.1 to 13.5, and if a single antigen at only one concentration is considered, then these ten patients gave a mean S.I. of 2.3 $\pm$ 4.0 (with the high S.D. being due to one very high result) to 9µg/ml of glycine extracted HPV-2 and a mean S.I. of 1.3 $\pm$ 1.1 to 4µg/ml of purified HPV-2. These results confirm the very low responses obtained in patients with long-standing warts, but, together with the increase in S.I. around the time of cure in two healthy laboratory staff with hand warts, suggested that the system was robust enough to use to investigate the CMI to HPV in patients attending the Colposcopy Clinic.

While it would have been easier to use a standard dose of

antigen, it was found in preliminary experiments that some people responded to a higher dose and others to a lower dose. Where PBM were limited, one or two concentrations of antigen in the range of 1-30 µg/ml were used and where cells were adequate, a wider range of concentrations covering four ten-fold dilutions of antigen was used. Antigen concentrations of 10 µg/ml and 30µg/ml were used in this laboratory in LPA studies with HSV-1 (Vestey et al., 1988a)while a concentration of 6 µg/ml for HSV-2 was found adequate by Iwasaka et al., (1983) in their studies. A standard dose of 1 µg/ml however was used by Drummond et al. (1985) in their LPA with BKV antigen and in other LPA in their laboratory (Donnenberg et al., 1984). The range of antigen concentrations available and tried in the current study therefore seems appropriate and no single concentration emerged as optimal.

Not only was there considerable variation in individual responses to different concentrations of antigen, but also in the response to different antigens. Of 92 colposcopy patients assayed in the first part of this study, 25% responded to at least one papilloma antigen preparation derived from BPV, HPV-1 or HPV-2 virions, while one patient whose cervical biopsy showed CIN III and koilocytes histologically, had an S.I. of 12.7 using CsCl-purified BPV as antigen and 14.7 using SDS.ME - disrupted BPV, another (also CIN III but without koilocytes) gave an S.I. of 1.3 with the CsCl - purified BPV and 10.7 with SDS.ME - disrupted BPV. It is perhaps surprising that any patient should react to purified virions of BPV, although Lancaster & Olson (1982) showed that 2/10 milk samples contained enough BPV particles to be detected in the electron microscope. SDS is toxic to lymphocytes and in some patients the use of SDS.ME disrupted antigen led to cell death, but occasionally as in the two patients mentioned above, dilution of the antigenic preparation eliminated this problem and revealed a specific response.

The lymphoproliferative response to non-specific mitogens reaches a peak after 3-5 days in culture, while stimulation due to viral

antigens such as HSV and CMV peaks around 5-6 days (Neill and Norval, 1985; Vestey et al., 1988a). Lee and Eisinger (1976) used a 6 day incubation in their papilloma virus assay, but in preliminary experiments in this study an 8 day assay was found to yield more positive responses (Table 7). This led to standard errors of 10-25% between quintuplicate wells and in later experiments, a 7 day incubation period was used with slight improvements in the standard error. The consistency of the response could not be significantly improved by feeding the cultures with additional medium at 5 days, (see Results Section 1.1), nor by adding IL-2 either on day 0 or day 4 (Results Section 1.41). Possibly the variations observed for different concentrations and different antigens reflect a borderline response due to a limited number of memory T cells in peripheral blood. This could explain why some patients with a past history of warts appeared to respond on one occasion but not on the next.

In the colposcopy patients, 91% of those who responded to PV antigens had a history of past or present skin warts, mainly on their hands and feet. The response was frequently to purified HPV-1 and HPV-2 virions and, since the majority of cutaneous warts are associated with these types, this result is perhaps not surprising. Nevertheless, detergent disruption is considered to reveal group specific epitopes, and it had been hoped that a response associated with cervical HPV infection might be revealed using this approach. Given that 75% of the patients tested remembered having had skin warts in the past, it would have been difficult to differentiate responses to cervical and cutaneous HPV infection and interestingly, the only three people who responded to antigens prepared by disruption in the absence of a response to purified antigen all gave a history of current wart infection (two with condylomata acuminata and one with a hand wart; cervical histology CIN III K+, no dysplasia past K+ and CIN III K- respectively). It can be concluded that the lymphoproliferative responses detected here using virion derived antigens reflect skin wart infection rather than an

association of HPV with CIN.

It is evident therefore that an LPA to study the association between cervical dysplasia and immune responsiveness to HPV requires typespecific antigens. Yet virus production in CIN lesions is very low and often undetectable. Not only would it be difficut to prepare sufficient amounts of viral antigen for satisfactory in vitro assays, but such antigens might be unlikely to illicit an immune response. On the other hand, early proteins from the E6 and E7 ORFs have been detected in both cervical carcinoma tissue and cultured cervical carcinoma cells (see Introduction, Section 2.32). Possibly with monoclonal antibodies sufficient quantities of E6 or E7 could be purified from bulk cultures of cervical cells, but currently it is simpler to produce fusion proteins by the transcription and translation of fragments of PV DNA containing the appropriate ORFs attached to an expression vector in competent bacterial cells, or to produce synthetic peptides from the know sequence data.

It was possible to test a few specimens with fusion proteins of L1, L2 and E2 of HPV-1 and to L2 of HPV-2. Because E2 proteins have not been detected in warts, it was thought that this fusion protein would act as a control for the later antigens particularly since contaminating β-galactosidase sequences were still present (Doorbar, personal communication). While only three positive responses out of 15 specimens from 9 patients were obtained, some agreement with the wart history given was found. One patient responding to HPV-L1 had had a plantar wart 10 years previously, one responding to HPV-2 L2 had long standing mosaic plantar warts while the highest response (S.I. = 29.0 to HPV-2 L2, see Table 17) came from a colposcopy patient with a past history of warts and a current thumb wart. More highly purified proteins removing the problems of β-galactosidase contamination and residual SDS toxicity might therefore be suitable for specific LPA testing against those HPV types which produce a complete replicative cycle, and would be worth trying particularly in patients with current

active lesions or in those whose warts had recently resolved. The recent findings by Stanley et al. (1988, personal communication) that a monoclonal antibody against HPV-16 L1 stained nuclei of koilocytes in cervical lesions containing HPV-16 would suggest that an HPV-16 L1 fusion protein might be worth trying in colposcopy patients.

Fusion proteins of E4 and E6 of HPV-16 and of E6 of HPV-18 were used in the study reported here. Eight positive responses to highly purified E6 proteins were recorded in 48 colposcopy patients and none in a group of 15 age matched healthy female members of staff (Fig 13), but the extent of proliferation was slight and of the five positives who were also tested with control antigen, only two gave S.I. more than twice that obtained with control antigen alone. While protein products of E6 have been found in cervical tumours and in transformed cervical cell lines (see Introduction, Section 2.32), they are not always detected (Lehn et al., 1985; Pater and Pater, 1988) and the E6 polypeptide may be expressed only at very low levels (Banks et al., 1987). Recent work suggests that the E7 protein is abundant although it has a half-life of about 1 hour (Smotkin and Wettstein, 1987). Nevertheless its abundance suggests it may be a better target for immune responsiveness and it would be interesting to test E7 fusion proteins in a similar assay.

The protein product of E4 is expressed in large quantities in HPV-1 warts (Croissant et al., 1985; Doorbar et al., 1986) but its significance in other HPV infections is unknown. Although an E4 protein of MW 10,000 was reported in CaSki cells (Seedorf et al., 1987), little evidence was found in the current study for immune reaction to HPV-16 E4. Slight stimulation occurred at 3 or 5 days of culture in 5/25 colposcopy patients and 1/15 controls when the control β-galactosidase fusion protein was used as antigen (Fig 14 and Table 20), but lymphoproliferative responses to the specific HPV-16 E4 sequence were not recorded after 7 days in culture.

Although the positive responses obtained in this study with fusion

proteins were weak and few, they are encouraging enough to suggest that HPV infection does produce antigens to which T cells can respond. By using only highly purified fusion proteins from the most appropriate ORFs, (such as L1 or L2 for productive infections, E4 for HPV-1 infections or E7 for HPV-16 containing lesions), more positive responses might be obtained.

It is of course possible that fusion proteins do not have the correct conformation to replace natural HPV determinants in in vitro tests and synthetic peptides from within HPV-16 L1 and E6, predicted on the basis of structural similarities with previously defined T-lymphocyte determinants (Rothbard and Taylor, 1988) have been tried in LPA. Women with CIN I-III and healthy donors having no apparent HPV-associated disease have responded, with individuals of different HLA-DR types appearing to respond to different regions of HPV-16 L1, depending on the peptide's ability to bind to a particular MHC allele (Dr. George Strang, personal communication). This approach, based on related studies to investigate CMI to influenza virus (Townsend et al., 1986) is well worthy of further study.

Since the majority of T cells interact with altered cell surfaces, the response of T cells to potentially HPV-infected cells was studied using a glycine extract of cervical tissue in 18 colposcopy patients (Table 17). Only one patient responded by giving an S.I. to an extract prepared from a biopsy of abnormal tissue which was between 3 and 4 times higher than the S.I. obtained with an extract of normal cervical tissue. It would have been useful to have known the HPV status within all biopsies used in this part of the study, but this was not possible. It would also have been valuable to have obtained normal and abnormal pieces of tissue in all cases to eliminate reactions to other foreign antigens in the tissue. Paired biopsies were however only available from three patients. Nevertheless it would be interesting to extend these investigations to study extracts of cervical tissue with and without HPV infection from the same patient. It is of note that the

patient who gave a positive response also responded to HPV-16 and HPV-18 E6 fusion proteins in LPA six months later and indeed one year later, clinical examination showed no dysplasia but revealed inflammation and infection with Candida in the cervix. One might speculate that a CMI response to infected cervical cells led to the release of early antigens of HPV-16, and the development of responding T cells, detectable in vitro at a later date. Adequate destruction of the infected cells would explain the lack of dysplasia at the most recent visit. However the presence of Candida infection suggests careful follow-up is required, in case the local immunosuppression associated with Candida infections (Witkin, 1987) should allow HPV infection to take hold once more.

A complex network of co-operative interactions mediated by secreted molecules is involved in any cell-mediated immune response and includes interleukins, helper and suppressor factors and interferons. Interleukin-1, a potent chemoattractant for T cells, is secreted by macrophages or dendritic cells. It triggers activation and proliferation of not only T and B cells but also other cells such as osteoblasts and fibroblasts, resulting in wide-ranging effects in vivo including fever and wound-healing. In in vitro assays such as the LPA used here where T cells are already in close contact its effects are less likely to be observed. On the other hand, IL-2 is essential for the continued proliferation of activated T cells, with $CD4^+$ activated by IL-1 being the major source of IL-2, but with each effector class of T cell being capable of clonal expansion in its presence. (Watson et al., 1983).

Certainly the addition of IL-2 to PBM in the absence of antigen produced stimulation with S.I.$\geq$2 in 32/68 people tested showing that many had activated T lymphocytes already present in peripheral blood. When compared with the background IL-2 response, however, specific stimulation due to HPV-1 or HPV-2 antigens was noted in 11 specimens (Table 22) and only in two of these was a positive S.I. to HPV obtained without added IL-2. Both of these specimens came from patients with current warts : one from a colposcopy patient with a thumb wart and the other from a patient with recalcitrant mosaic warts. Interestingly, resolution did not occur in the latter during the six months of

observation of the trial of inosine pranobex. The time of resolution of the wart in the colposcopy patient is unknown.

In contrast, fifteen other specimens which gave a positive S.I. in the presence of HPV antigen were not significantly boosted by the addition of IL-2. Nevertheless these results show that in some pople (9/68, 13% in this study), T cells can be specifically activated by the presentation of HPV-1 or HPV-2 capsid antigens and that proliferation can be amplified to significant levels by the addition of IL-2. In the remaining people, despite a past history of skin warts from most of them, insufficient T cells must have been activated for IL-2 to be effective. It was therefore considered that alterations in APC might yield more positive responses.

An alternative source of APC in the form of autologous cervical epithelial cells disaggregated from biopsy tissue by collagenase-dispase treatment, was included (Table 24), but this was largely unsuccessful in the 13 specimens in which it was tried. Certainly in a study of the presentation of HSV antigens to lymphocytes, human epidermal cells separated from the dermis by suction blister and dissociated with trypsin were more potent than PBM in inducing lymphoproliferative responses to HSV-1 antigens in LPA using PBM (Braathen and Thorsby, 1983; Bagot et al., 1985; Vestey et al., 1988b). In the more recent papers, the HSV-1 antigen was UV inactivated and epidermal cells were incubated in its presence before being added as stimulator cells to PBM (Bagot et al., 1985) or the non-adherent PBM population (Vestey et al., 1988b). Prior incubation of the cervical epithelial cells in culture medium containing HPV antigens might have been a useful addition to the method used here. Similarly, the use of epidermal cells from suction blisters and antigens from cutaneous HPV types in assessing the lymphoproliferative capacity of PBM in patients with present or recently resolved skin warts would be well worth trying. The results obtained in such a study would provide a better basis for examining the response to proteins from HPV types associated with cervical dysplasia.

In HSV infection of the skin, the virus becomes latent in sensory neurones in the area of local innervation of the skin in which the primary infection occurred. Reactivation of the latent virus can lead to fresh infection of epithelial cells (recurrence) with the development of a clinical lesion (recrudescence) in some cases (Wildy et al., 1982). Recurrent lesions in guinea pigs were shown by Iwasaka et al. (1983) to be associated with the induction of suppressor cells and soluble suppressor factors. The suppressor factors could be detected in the supernates of LPA and were effective in inhibiting both the lymphoproliferative response to HSV-stimulated immune cells and Con A-stimulated normal lymphoid cells. In the present study it was only possible to test on one occasion the effect of LPA supernates collected on days 4, 5 and 7 of the LPA on autologous PBM exposed to HPV antigens or Con A. No response was obtained. However Iwasaka et al. (1983) added supernate at a final concentration of 50% in their test while in the present study it was added at a much lower concentration (5%). It would be well worth repeating this assay with much higher concentrations of supernate, but the logistics of obtaining large enough volumes of blood on at least two occasions in close succession from people who had had previous positive results were daunting.

The effect on the Con A response of adding papilloma antigen at the same time as Con A was assessed in 13 specimens (Table 25 and Fig R5). In 4, the S.I. with Con A was more than doubled by the addition of HPV-16 E6 when analysed at 7 days. This was thought to represent a slowing-up of the Con A response due to an inhibitory effect in the presence of PV antigen and certainly when the effect was analysed at earlier time points, the S.I. to Con A + HPV-16 E6 or HPV-2 at 3 days was generally less than the response to Con A above. Nevertheless, the peak response to Con A was not altered. Little effect was found on adding HSV and HPV antigens together (Fig 16). It seems likely that the time scale of such experiments was too short. It would have been better to have allowed a potential suppressor

response to develop and then added cells from the first experiment to fresh autologous PBM in the presence of antigen. Such a method was used to detect suppressor cells induced by lepromin in patients with lepromatous leprosy (Mehra et al., 1980) and by BCG antigen in healthy individuals vaccinated with BCG (Mustafa and Godal, 1985). In the latter study, the induced suppressor cells appeared to inhibit BCG induced IL-2 receptor expression on the fresh T cells. As with supernates, such experiments with HPV would require careful planning and willing volunteers with consistent positive responses.

The virus-specific suppression of immunity to HSV-2, resulting in clinical recrudescences has been suggested to be $CD8^+$ mediated (Sheridan et al., 1982) and to involve the production of suppressor factors (Sheridan et al., 1985, 1987). The suppressor factor interfered with IFN and IL-2-mediated NK cell activity and while the prostaglandin, PGE was necessary, it was insufficient to account for this activity (Sheridan et al., 1985). It has recently been shown in this laboratory that the removal of $CD8^+$ lymphcytes by panning increased the S.I. obtained in LPA during recrudescence of HSV-1 facial lesions (Vestey et al., 1988a). In the three specimens on which this was tried in the current study, no increase in S.I. was observed by the removal of $CD8^+$ cells although the Con A response was markedly reduced at 3 days by $CD8^+$ depletion and potentially restored by their replacement. However panning is only partially successful in removing $CD8^+$ cells. In 9 subjects tested in this laboratory (Dr. James Vestey, personal communication), panning reduced the $CD8^+$ percentage in PBM from an average of 22% to 14% while reconstitution in the ratio of 3:10 increased the percentage to 41%. Cell sorting by EPICS analysis was found to be considerably more effective, reducing the $CD8^+$ population to approximately 2%. With such efficient depletion methods it would be worth repeating these experiments using different HPV antigens.

Flow cytometry was also used to investigate the type of T cell present at the end of lymphocyte culture in the presence of HPV

antigens (Results, Section 1.44). The method developed included staining the cells in the plates in which they had been cultured. This was not only straightforward and practical to perform, but it was more economical with cells, requiring 2×10^5 cells per well compared with 5×10^5 cells per tube. However, it became apparent that the cultivated cells were particularly fragile. Unless they were analysed immediately after staining, they tended to become disrupted and to lose attached stain. This fragility has been confirmed in further experiments in this laboratory (Mr. W. Neill, personal communication). The larger volumes of buffer used to wash the cells when they are stained in 3 x ½" tubes and the ease with which they can be resuspended might allow more consistent results to be obtained at the end of the culture period, more than compensating for the advantage of retaining the cells in the culture plate and the commensurate savings in reagents.

In two specimens there was a suggestion that $CD8^+$ cells were increased in the first few days of the LPA (Table 27). If this could be verified, it might suggest that the activation of a small number of memory T cells would generate an early $CD8^+$ response with the release of a suppressor factor inhibiting further proliferation without adequate proliferation of the $CD8^+$ cells for stimulation to be observed. In studies using PBM from patients with recrudescent genital HSV lesions, Sheridan et al. (1987) showed that an inhibitor of lymphokine activity was produced within 24 hours of culture in the presence of UV-inactivated HSV, either as a product of activated $CD8^+$ cells (OKT8 and OKIa markers) in the presence of monocytes (OKMI marker) or of a single cell expressing all three surface markers. Lehner et al., (1982) also found that in Rhesus monkeys immunised with streptococcal antigens, suppressor cells reached a peak at 2 days with maximal suppressor factor secretion on day 3, while helper cells peaked at 4 days. In addition, Fox et al. (1986a) noted that when $CD8^+$ cells were cultured in PWM for 6 days in the presence of IL-2, they exhibited very little

proliferation despite secreting suppressor factor. Additional suppressor cell growth factors in addition to IL-2 from cells of $CD4^+$ lineage were required for proliferation. The kinetic relationship of immunoregulatory circuits in in vitro tests, while different for different antigens, needs further exploration and this will be particularly difficult with HPV antigens where reactions are weak and difficult to elicit.

2. Possible Pathways of CMI Development in HPV Infection (see Fig 33)

T cell responses are triggered only if antigen is presented properly by APC. For example, in rabies infection, the virus may hide in neuronal cells for considerable periods before the cells are destroyed, and antigen is released and then taken up by APC. In the intervening period, no CMI responses are detectable. (Zinkernagel et al., 1985). In HPV infections, there is increasing evidence that in common warts, in non-regressing cutaneous warts of various types and in HPV and CIN+HPV lesions of the cervix, Langerhans cells are reduced in numbers and in morphology (see Introduction, Section 4.322), suggesting that their ability to act as APC will be diminished. A lack of presentation of antigen would prevent most of the cascade of reactions dependent on IL-1 production by APC and IL-2 production by helper T cells (reaction 1, Fig 33).

Nevertheless some stimulation of Th (reaction 2) does frequently occur in HPV infections since the induction of B cells is usually dependent on T cell help (except for the rare T-independent antigens) and specific HPV antibodies are detected (reaction 4) in approximately 50% of any group tested (see Introduction, Section 4.1). Furthermore massive lymphocyte infiltration with spontaneous resolution of whole crops of HPV-3 induced plane warts would support the occurrence of reactions 5 and 7, and 6 and 8. Cytotoxic T cell responses could explain the speed with which the resolution of plane warts occurs. Indeed the satellite cell necrosis observed by Iwatsuki et al. (1986) in

Fig. 33 Possible pathways of CMI development in HPV infection

Abbreviations

LC = Langerhans cell

Th = Helper T Lymphocyte

Ts = Suppressor T lymphocyte

Tc = Cytotoxic T lymphocyte

TDH = Delayed hypersensitivity T cell

B = B lymphocyte

MΦ = Macrophage

Ker = Keratinocyte

NK = Natural killer cell

PC = plasma cell

∩ = MHC Class I

Π = MHC Class II

Y = antibody

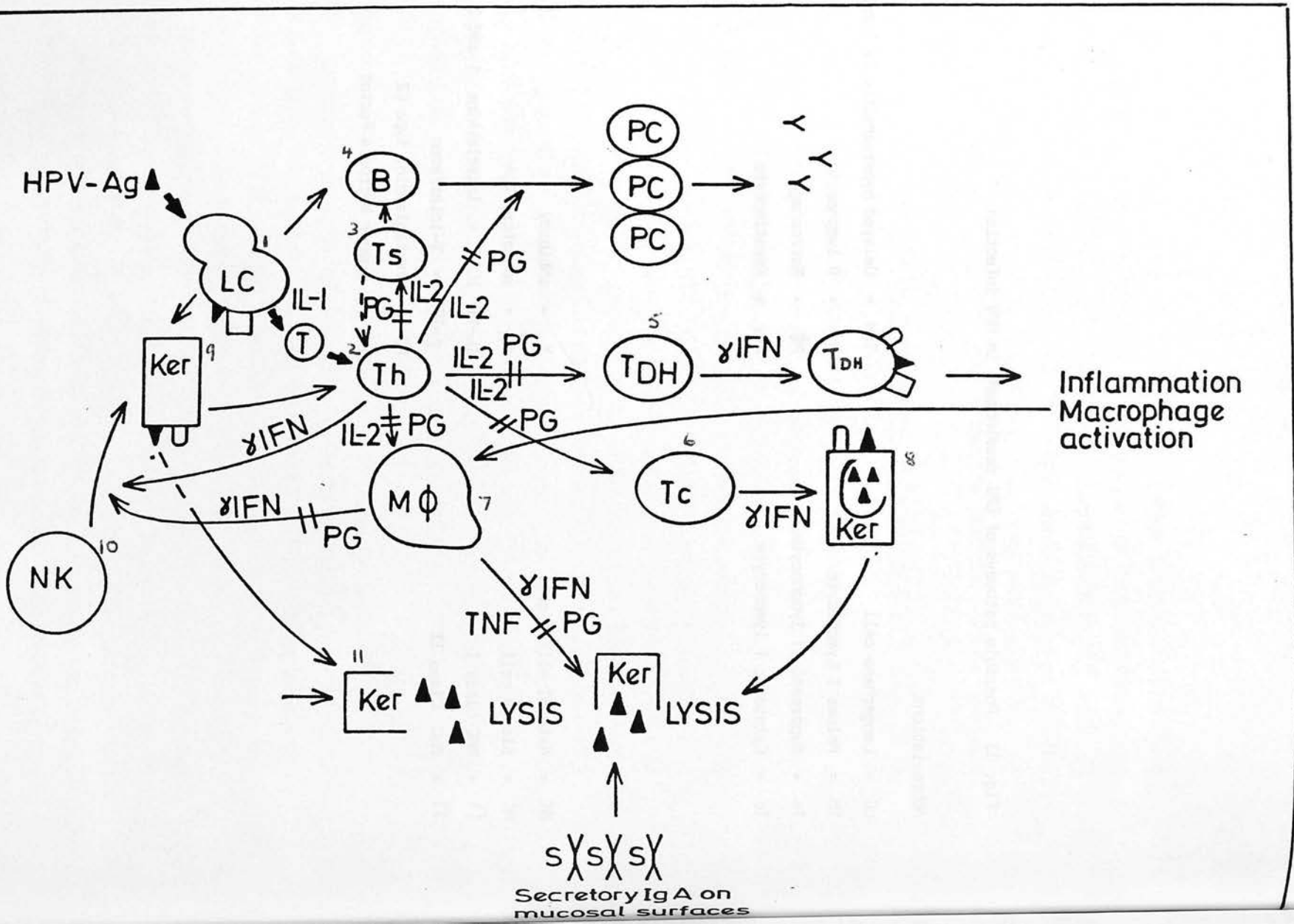
sY = secretory IgA

IL-1; IL-2 = interleukins 1 and 2

γ-IFN = γ-interferon

PG = Prostaglandin, type E2

TNF = Tumour Necrosis Factor


HPV-Ag
LC
1
B
4
Ts
3
PC
PC
PC
PG
IL-1
IL-2
IL-2
T
2
Th
IL-2
PG
IL-2
TDH
5
γIFN
TDH
Inflammation
Macrophage
activation
Ker
9
γIFN
IL-2
PG
PG
6
Tc
γIFN
Ker
8
MΦ
7
γIFN
PG
NK
10
γIFN
TNF
PG
11
Ker
LYSIS
Ker
LYSIS
Secretory Ig A on
mucosal surfaces

such regression is the hallmark of a cytotoxic reaction, although these workers concluded that, since the cellular infiltrates contained cells other than $T_{s/c}$ cells, regression was a complex phenomenon with interleukins and IFN modulating a DH-like reaction, characterised by the associated inflammation during regression. The persistence for several years of T_{DH} to HPV has been shown by in vivo skin testing of several groups of patients (Viac et al., 1977a, and 1978) with 75% of patients with past warts, 56% of patients with present warts and only 7% of control with no wart history showing positive reactions (Thivolet et al., 1977).

In order to detect specific T_c reactions in vitro, it may be necessary to amplify them by clonal expansion in a mixed lymphocyte/target cell interaction using mitomycin-C treated infected cells in culture with autologous PBM for several days (Roitt et al., 1985) followed by an isotope release assay to show T_c. It would have been interesting to have tried this approach in colposcopy patients in whom adequate biopsies and matching blood samples were available. This kind of approach was taken by Tagami et al. (1985) when they added PBM to one-week old explants of wart-derived epidermal cells. Affinity for infected cells but not for normal cells was shown by autologous PBM and subtyping of the cells involved was said to be underway, with $T_{s/c}$ ($CD8^+$) cells outnumbering $T_{h/i}$ ($CD4^+$).

When HPV infects the basal cell of the epidermis it does so in tiny quantities, causes no CPE in these cells and, with limited transcription even in suprabasal layers, it may appear in vivo as weakly antigenic. Exposure of the immune system to such small doses of weak antigens produces tolerance, maintained by the action of suppressor cells which are triggered at a much lower antigen concentration than helper T cells (Roitt et al., 1985). The need to develop suppressor cellular circuits to regulate the immune response is not necessary for acute cytopathic viruses where the immune response fades away after elimination of viral antigen (Zinkernagel et al., 1985),

but HPV does not fit into the category of an acute cytopathic virus. Within the suppressor circuit, some T_s may become memory cells capable of being reactivated following subsequent contact with antigen and maintaining a tolerised state. The finding of $CD8^+$ cells in spontaneously regressing plane warts and described above does not support the concept of T_s memory cells subsequently suppressing the response to future contact with HPV-3 since re-infection with HPV-3 after such regression is unknown (Tagami et al., 1985). Rather, it supports the presence of cytotoxic cells destroying infected cells with the release of viral antigen stimulating a peripheral antibody response which offers protection from subsequent attack by the same virus. Although other HPV types do not appear to act in such a dramatic way as HPV-3, Becker et al. (1985) have shown that oral papillomas contain a local increase of $CD8^+$ cells to reduce the T4:T8 ratio to 1 together with an increase in macrophage numbers.

The administration of the immunosuppressive drug, cyclosporin A, lowers the threshold for induction of tolerance, particularly when given at the same time as antigen, and blocks the cell sequences normally triggered by antigen (Roitt et al., 1985). While it suppresses IL-1, IL-2 and γ-IFN production, T_s production, by an IL-2 independent pathway is spared (Bennett and Norman, 1986). It is easy to imagine, then, that with continuing treatment with cyclosporin A and exposure to ubiquitous HPVs, renal transplant patients will become more tolerant and this can explain the more rapid development of HPV lesions in them. In addition, steroid treatment with or without azathioprine exert an effect on T_{DH} cells further increasing immunosuppression. (ten Berge et al., 1981).

Suppressor T cells can be assayed by the capacity to inhibit primary lymphocyte transformation by lectins (Roitt et al., 1985). This was tried in the current study, but although a shift in the Con A response was obtained in the presence of HPV antigens in laboratory personnel whose lymphocytes had proliferated in the presence of such

antigens, a true inhibition was not observed. This may be a reflection on the sensitivity of the system and is worth repeating with blood from people giving good stimulation with skin HPV antigens and having a history of warts in the recent past.

Both suppressor and cytotoxic cells are currently detected by the same marker ($CD8^+$) and this has led to controversy over the very existence of antigen specific suppressor cells. Nevertheless, the phenomenon of suppression undoubtedly exists and Batchelor et al., (1989) have recently suggested that the functional phenotype of $CD8^+$ cells was dependent on the idiotype of the presenting T-cell receptor. Martz and Howell (1989) have recently hypothesised, furthermore, that too much importance has been attached to the lytic function of cytotoxic cells and they suggest that T_c are the specific viral control cells requiring direct contact with infected cells and capable of halting viral replication prelytically or by lymphokines in the absence of cytolysis.

Natural immunity in the form of NK activity (reaction 10, Fig 33) in PBM against specific HPV-containing target cells has been shown to occur (Malejczyk et al., 1987), both in patients with Bowenoid papulosis and anogenital carcinoma, and in EV patients with skin tumours (Malejczyk et al., 1989). NK cells are effectors of natural immunity in the absence of prior stimulation and they play a role in regulating tumour development as well as in combatting virus-infected cells. It is therefore not surprising to find increased NK activity against HPV infected cells and it would be interesting to extend these observations for example, to patients with common warts or to renal transplant recipients with unusual HPV lesions.

Additional factors have to be considered in the progression of the response to HPV infection. It is generally accepted that virus enters the skin through a wound, yet in the healing of that wound, immune cells are active and may influence the response to the virus. T_h cells are found in the dermis in the early stages after damage, and

LC in the epidermis are activated by both intrinsic (serum proteins) and extrinsic (bacteria, viruses) factors (Morhenn, 1988).

The production of IL-1 by LC leads to proliferation of dermal fibroblasts, the release of somatomedin C and the resultant mitosis of keratinocytes to effect a repair. The activated T cells produce γ-IFN which can act on keratinocytes to induce MHC Class II expression (mainly HLA-DR) enabling the keratinocytes to act as APC themselves and augmenting the local immune response (Morhenn and Nickoloff, 1987; see reaction 11 Fig 33). However, γ-IFN has the opposing property of augmenting the levels of prostaglandin E_2 (PGE_2; see reaction 9 Fig 33) which can switch off IL-2 production (Nickoloff et al., 1986). An increase in prostaglandins could therefore diminish the immune responsiveness to HPV infecting at the time of wound repair. Such an action could explain why recalcitrant warts are so common on the hands which we subject to frequent minor injuries and might be particularly relevant in HPV infections of the cervix where repeated epidermal damage and repair can occur. In addition, the inhibitory effect of prostaglandins could be augmented cyclically in women by hormones acting like gluco-corticosteroids which similarly suppress regulatory proteins such as IL-1 and IL-2 (Gifford and Flick, 1987). Both prostaglandins and glucocorticoids can also inhibit tumour necrosis factor (TNF), an important effector molecule in the non-specific tumoricidal activity of activated macrophages. In view of the recent evidence that HPV-16 transcription and transformation can be increased by dexamethasone (Gloss et al., 1987; Pater and Pater, 1988) it would appear likely that hormonal influences could both positively promote HPV infection and at the same time switch off an immune response to it.

The cellular nature of CMI can be more definitively analysed immunocytochemically with the wide range of monoclonal antibodies now available and indeed, in a study in Cambridge of HPV infection and

dysplasia, a panel of 23 different monoclonals of immunological and viral markers has been employed (Dr. Margaret Stanley, personal communication). Nevertheless, such studies need to be accompanied by functional analysis of the involved purified cell populations, but this presents almost insurmountable logistical problems, ethically and in terms of the amount of material available from an individual patient. The result is the current plethora of data which cannot yet be put together in a cohesive way. Figure 33 is an attempt to show the reactions described above which might be involved in the response to HPV infection.

Tumours which develop as a result of HPV arise because infected cells have escaped the destructive mechanisms of immunological controls and been encouraged to grow by tolerance, by suppression of T and B responses, by soluble factors and in some cases by genetic influences where there is a basic failure to induce an effector T cell response of any kind.

The defects of non-specific CMI noted in many patients with warts through weaker intradermal tuberculin tests (Brodersen et al., 1974), depressed LPA responses to mitogens (Morison, 1975c) and decreased T cells levels (Chretien et al., 1978) have prompted the use of immunostimulants in their treatment. Regression of warts after treatment with immune adjuvants such as DNCB and vaccination with autogenous vaccine have both been reported (Chardonnet et al., 1985). For example, repeated intradermal innoculation of formalin-inactivated HPV particles led to the acquisition of specific cellular responsiveness and an accelerated regression in 10/22 patients with multiple or recurring warts (Viac et al., 1977a). Interferon has been successfully used in the treatment of various types of warts. Topical IFN-α was shown to be effective for condylomata (Scott and Csonka, 1979) and systemic IFN-α can reduce the growth rate of laryngeal papillomas (Steinberg and Abramson, 1985).

More recently, inosine pranobex has been used as an

immunopotentiating agent in the treatment of genital warts. Two French reports suggested that inosine pranobex was remarkably effective in treating vaginal and cervical condylomata (Malgouyat, 1983) particularly when used in conjunction with CO_2 laser therapy (Sadoul and Beuret, 1984) and a more recent report suggested it was especially effective in genital warts of long duration (Mohanty and Scott, 1986). The French trials, however, were not controlled trials and long-term treatment was required. The data is not yet analysed from the on-going double-blind placebo-controlled study of the use of inosine pranobex ("Imunovir") being carried out in the Department of Dermatology here in patients with recalcitrant common warts. Nevertheless, in the group of 10 patients on whom LPA was carried out, 3 out of 5 of the treated group were cured within 3 months whereas none of the warts in the placebo group had resolved, although two showed slight improvement. However, by 7 months after the commencement of treatment, 4 patients who had received the active compound and 3 who had received placebo, were cured. It will be interesting to see if the early improvement with "Imunovir" is reflected throughout the trial group. A high regression rate in the treated group would support the concept that, in many cases of HPV infection, the cells of the immune system are primed, but not adequately and the cascade of subsequent events is not generally triggered, but when the response is amplified by immunotherapy, it can be effective in bringing about resolution.

3. Humoral Responses to HPV Antigens

Early studies showed that antibody could be detected in 25-50% of people with current or past warts with higher percentages of positives (67-100%) when people with regressing lesions were considered (see Introduction; Section 4.1). It was expected that recent advances with more sensitive techniques such as ELISA or Western blotting, coupled with the use of type specific antigens would lift this figure

much higher. Nevertheless, Pfister and zur Hausen (1978) found only 50% of youngsters and 45-55% of adults with cutaneous warts, 53% of patients with genital warts and 40% of patients with various carcinomas to have antibodies to HPV-1 using ELISA and RIA tests; Pyrhonen et al. (1980) found 44% of patients with common warts to have IgG antibodies and 71% to have IgM both measured by ELISA; Kienzler et al. (1983) found only 39% of patients with myrmecia to have antibodies to HPV-1, and Li et al. (1987a) found 60% of women attending a colposcoy clinic to have antibodies to HPV-6b L1 fusion protein by Western blotting.

In the current study too, an approximate 50% positivity rate was found in each group with SDS.ME - disrupted HPV antigens. Overall, 51.1% of colposcopy patients tested had antibodies to HPV-1, HPV-2 or both and within each grade of dysplasia, approximately 50% of patients had antibodies irrespective of whether koilocytes were present or not. This finding, supported by a history of skin warts in over 70% of those patients with positive ELISA results suggests a response to an HPV other than that producing the cervical lesion and supports the concept that a type-specific reaction to a skin wart infection is being detected in these patients.

While disruption of virions by SDS.ME is thought to reveal a group-specific antigen to which animal antisera with cross-reactivity can be prepared (Jenson et al., 1980), it is possible that in vivo these internal common antigens may not be exposed to the immune system. That the ELISA described here does indeed detect type-specific antigens is shown by the fact that some patients reacted with HPV-2 and not with HPV-1 (Table 30). The 64 patients who had antibodies to both HPV-1 and HPV-2 had probably had infections caused by both viral types, although some degree of cross-reactivity due to the use of disrupted particules may be a contributory factor. No cross-reactivity, however, was apparent with disrupted BPV. 55 patients gave negligible absorbancies (< 0.13) with disrupted BPV used at similar concentrations

to the HPV-1 and HPV-2 antigens where absorbancies ranged from 0.23 to 1.21 for HPV-1 and from 0.24 to 0.56 for HPV-2. These variations in absorbancies may reflect differences in exposure to antigen. In HPV-1 lesions, much more virus is produced and, since resolution is often accompanied by haemorrhage and thrombosis into the substance of the wart (Bunney, 1982), it is assumed that antigen is released with subsequent development of antibody. HPV-2 lesions produce much less virus as determined by EM examination and rarely show such impressive regression. Exposure to BPV would be much less.

These results are at variance with the results reported by Baird (1983) using disrupted BPV-2 particles in a similar indirect ELISA. Baird reported higher antibody levels as measured by higher absorbancies in the sera of more than 90% of patients with anogenital warts or invasive cancinoma and in more than 60% of patients with CIN. The comparison was made with the absorbancies of sera from a group of children and from a group of symptomless adult females. However, even in his cancer group who showed the highest levels of antibody the mean absorbance was just over 0.2 and it is questionable whether such small differences and low absorbancies can be considered significant.
A similar study was carried out by Beiss et al. (1987) in an effort to extend Baird's work. 38% of women with flat condylomas or CIN were shown to have antibodies to disrupted BPV-2 when compared to subjects with no history of genital lesions or abnormal cervical smears.
However in this study the cut-off for positivity was only one standard deviation above the mean absorbance of the control group. Had the cut-off been set even at 2SD above the mean, only 18% of the patients would have been positive. It seems unlikely that the detection of a reaction to genus-specific antigens not exposed on the virion surface should have much bearing on the presence of cervical HPV lesions per se, but rather reflect the ability to mount an antibody response to the normally hidden common antigens of any HPV type. This suggests that processing of HPV perhaps with lysosomal degradation must occasionally occur.

ELISA systems can be amplified by increasing the number of layers applied. For example, the enzyme-linked second antibody can be replaced by a biotinylated antibody and binding of this to the primary antibody-antigen amplified by enzyme linked streptavidin or even further with streptavidin-biotin bridges. Since streptavidin can bind four biotin molecules, this method allows three-fold amplification.

It is perhaps naive however to expect that by increasing the sensitivity of the test, higher detection rates of antibody to HPV antigens will be found. If the cellular responses described in the previous section are valid for HPV, then there will be many cases of infection in which the host response is cut short and a detectable B cell response will not follow.

Most of the ELISAs carried out in the current study used virion derived HPV antigens. Indeed the few attempts to use fusion proteins were wholly unsuccessful and other workers have also found problems with the method (L. Gissmann; L. Banks, personal communications). It is likely that the fusion proteins from HPV-1 and HPV-2 (HPV-1 L1, L2 and E2 and HPV-2 L2) were not sufficiently pure to give specific results. Jenison and colleagues (1988) noted that extensive preabsorption of sera with large amounts of bacterial lysate was necessary to overcome the problem of antibodies directed against E.coli proteins. Although the HPV-16 and HPV-18 E6 fusion proteins provided here were highly purified and devoid of contaminating sequences, too little was available to develop a reproducible assay.

Immunoblotting systems using filter-bound HPV fusion proteins reacted with serum specimens and detected by a variety of systems comparable to those used for ELISA may prove more useful. Already, antibodies to the late gene products of HPV-6b have been detected in patients with genital warts and to early gene products occasionally (Li et al., 1987a; Jenison et al., 1988; see Introduction, Section 4.1). Work in this area is progressing in several centres, but care will be required in interpreting the data obtained since the conformation of

immunoreactive epitopes may be destroyed in Western blots. In addition, very careful definition of the particular fusion proteins used will be required if responses to type specific and genus specific epitopes are to be distinguished.

While the systemic humoral response may only be detected or even activated in approximately 50% of infected people, the local antibody response particularly to HPVs infecting mucocutaneous sites may be more relevant. Most B lymphocytes at mucosal surfaces are dedicated to IgA synthesis, and much IgA in the mucosa exists in polymeric form to protect it from proteolytic enzymes (Underdown and Schiff, 1986). To transport the IgA from the site of synthesis to the mucosal lumen requires the synthesis by epithelial cells of a 70,000 MW transmembrane glycoprotein termed secretory component, which binds to the IgA molecule almost irreversibly and then acts as a shuttle to conduct it across the epithelial barrier. When released, IgA has much of the secretory component still attached making it more able to resist proteolytic cleavage and more resistant to pH changes (Underdown and Schiff, 1986).

The existence of a local secretory immune system in the endocervix providing the major source of immunoglobulins in cervical mucous was shown by Rebello et al., (1975). High levels of sIgA to chlamydia and HSV were found using a radio-immuno assay in cervical secretions from patients with malignant atypia or dysplasia (Kalimo et al., 1981). Interestingly, the persistent production of anti-chlamydial IgA in the secretions supported the presence of latent or chronic infection while in HSV infections, sIgA appears to be an important factor in reducing the duration of cervicovaginal excretion of HSV (Merriman et al., 1984). It therefore seemed valuable to look for specific sIgA to HPV in cervical secretions from colposcopy patients. The simple but sensitive indirect ELISA recently described by Fox et al., (1986b) to detect sIgA in tears was adapted for use with cervical secretions. Although high absorbancies were obtained in one third of

the specimens tested, when compared to the absorbancies obtained with matching plasmas, no quantitation of the results was carried out. Several of the specimens contained considerable amounts of mucus and one or two were slightly bloodstained. Removal of the mucus by enzymic degradation, or by spinning through or by including a high concentration of salt followed by filtration through an inert sea sand and glass wool bed, might have been worth including in the preparation of the specimens. This latter method apparently gave good recovery of soluble proteins from cervical secretions (Fleetwood et al., 1984). It would also have been useful to have estimated the protein content of each specimen in an effort to standardise the specimens used. Perhaps an ELISA based on an anti-secretory component-capture assay would have allowed more specificity despite variation in samples. Anti-secretory component coated on to the wells of the plate would capture sIgA in the specimen some of which, if specific for HPV antigens, could be detected by successive incubations with disrupted HPV, rabbit anti-HPV and enzyme-linked anti-rabbit serum. Detection of sIgA is an area worthy of further study, in order to understand when and how local immune responses to HPV develop, and interestingly, a very recent publication reports the use of an indirect ELISA for HPV-IgA as a useful marker of the presence of CIN+HPV (Dillner et al., 1989).

4. In situ Detection of HPV

One of the first reports of hybridisation to detect viral DNA came from Orth's laboratory where it was found that the replication of CRPV in rabbit tumours, as shown by the binding of an ^{3}H labelled probe, was restricted to the upper layers, with increased grain density as keratinisation of the cells progressed (Orth et al., 1971). A similar picture was shown for human cutaneous warts (Orth et al., 1980).

Improvements in the efficiency of ISH by increasing the diffusion of probe, by optimising the temperature of hybridisation, by stabilising the hybrids during post-hybridisation treatments and by hybridising in the presence of excess c DNA (Brahic and Haase, 1978) led to much wider application of the technique.

Non-radioactively labelled DNA probes, prepared by nick translation in the presence of biotinylated d-UTP were first described by Langer et al. (1981), but to prevent steric hindrance in detecting bound biotinylated probes, a spacer arm between biotin and the pyrimidine ring had to be inserted. Various lengths of spacer arm were tried and while an increase from 4 to 11 atoms enhanced target detection four-fold, further increase in length were no more efficient and an 11 atom linker was selected as optimal (Leary et al., 1983). Probes labelled in this way were successfully used to detect viral genomes in cultured cells and in paraffin embedded tissues (Brigati et al., 1983). It was noted that to achieve efficient and reproducible hybridisation, consistently good tissue adhesion and a high degree of premeability of cells, while maintaining morphological integrity, were essential.

These two aspects have been frequently mentioned as problem areas in subsequent papers on ISH and required close attention in the study undertaken here. An almost complete loss of sections occurred on untreated slides, for example, and even slides coated with poly-L-lysine, which is frequently used in immunocytochemistry to improve adhesion (Huang et al., 1983), were often inadequate. The chemical silanation of slides coated with Denhardt's solution (Tourtellotte et al., 1987) was efficient and no sections at all were lost from slides used within 8 weeks of activiation. The effectiveness is due to strong covalent bands between aminoalkyl groups and aldehyde which can withstand repeated washings with inorganic solvents. Nevertheless, it is a lengthy procedure and the quicker, simpler and cheaper method of immersing clean slides in a 2% solution of

3-aminopropyl triethoxysilane in acetone (Burns et al., 1987) or methanol (Maddox and Jenkins, 1987) was almost as effective and is therefore more likely to be adopted by other users.

A high degree of permeability is achieved by treatment of sections with 0.2M HCl and by proteinase K digestion. However it has been suggested that excessive acid may depurinate DNA (Pardue, 1985) and this stage is now being omitted by some workers (Syrjanen et al., 1987) but was included in the current study. The concentration of proteinase K found to be most effective in maintaining integrity of the sections was 0.2mg/ml, within the range recommended by Brigati et al. (1983). The activity of the enzyme will vary with the batch and the conditions under which it is stored and used, but it is interesting to note that Beckmann et al. (1985) required 4mg/ml of Pronase B, and Syrjanen et al. (1987b) noted significant increases in hybridisation signals by increasing the concentration to 1mg/ml with only a slight deterioration in cell morphology, when studying HPV in condylomata with biotinylated probes.

A simple detection method using antibiotin antibody followed by fluorescein-labelled conjugate (Brigati et al., 1983) has been used, but amplified systems such as anti-biotin or avidin followed by anti-avidin as primary and secondary antibodies, followed by preformed complexes in which many biotinylated horseradish peroxidase molecules are cross-linked to avidin in a three dimensional assay (Hsu et al., 1981) have been favoured (Brigati et al., 1983; Beckmann et al., 1985). To circumvent background staining due to endogenous peroxidase, alkaline phosphatase detection systems have been used (Ungar et al., 1986; Burns et al., 1987; Lewis et al., 1987; Syrjanen et al., 1987b) and indeed the only commercially available HPV typing kit utilises biotinylated HPV probes with an avidin/biotinylated alkaline phosphatase detection system (Vira type^{TM}; Gibco-BRL, Paisley).

The vitamin biotin is widely distributed in tissues, especially liver, kidney, adipose tissue and mammary gland. In addition, it is

synthesised by gut flora and excess is excreted. It might therefore be expected that non-specific attachment of the detection system could occur. However, endogenous biotin is not found in high levels in skin and it is thought that the thorough washing stages in hybridisation remove this small molecule (MW of biotin being 244). It is possible to block endogenous biotin by saturating with excess avidin followed by biotin, which, with only one binding site, is no longer available for further reaction (Wood and Warnke, 1981). In the current study, background staining in formalin-fixed paraffin embedded sections with biotin or its detection reagents was rarely a problem. The pattern of non-specific staining observed occasionally with bipolar nuclear staining and/or cytoplasmic staining of basal and parabasal layers was readily distinguished from the more regularly stained nuclei of cells of the upper layers when specific probes were used. By contrast, in current investigation of respiratory virus detection with biotinylated oligonucleotides, endogenous biotin has been found to be a problem in cultivated cells grown in serum-containing medium and fixed in paraformaldehyde. Use of the steroid hapten digoxigenin (Boehringer Mannheim) as an alternatve non-radioactive label may circumvent this problem.

More than ten years ago, Brahic and Haase (1978) suggested that reducing probe size to approximately 50 nucleotides should increase diffusion and efficiency of hybridisation three fold. In some studies with HPV-DNA detection probe size has been reduced by the incorporation of DNAse to the nick translation system, to 60-160 nucleotides (Naghashfar et al., 1985) and to 200-400 nucleotides (Stoler and Broker, 1986; Collins et al., 1988), but no comparisons with untreated probes have been carried out. An alternative approach exists for probes where the sequence of DNA is known, in the form of synthetic oligonucleotides. This approach to the diagnosis of viral disease was first suggested by Richman et al. (1984), but it has taken several years for evidence of its usefulness to be forthcoming.

Robertson et al. (1987) used a 50-mer probe complementary to the measles virus nucleocapsid gene to identify persistent measles in lymphocytes from patients with autoimmune chronic active hepatitis. A smaller probe (21-mer) was used by Lin et al. (1987) for the detection of hepatitis B DNA in serum by dot-blot hybridisation and was found to give an equally sensitive, but quicker and simpler assay than that using a conventional nick-translated cloned probe. The short probes anneal more rapidly to their targets (Meinkoth and Wahl, 1984). Indeed, Jablonski et al. (1986) suggested that short oligos to HSV and hepatitis B virus covalently and cross-linked to alkaline phosphatase reached maximum hybridisation to target DNA fixed on nitrocellulose filters in 15 minutes.

Many diagnostic virology or pathology laboratories do not have the expertise, large-scale bacterial culture systems and containment facilities required for the production of probes from cloned nucleic acid and the use of synthetic oligonucleotides should overcome this. Nevertheless, many problems in their use have still to be resolved. Probe length, for example, can be varied over a limited range, but what are the advantages and disadvantages of so doing ? The smaller the probe, the less mis-matching is permissible and therefore the more specific the individual probe. Thus, Sano et al. (1988) were able to distinguish hop stunt viroid of grapevine from hop stunt viroid of hop using a 15-mer, despite only a single nucleotide difference between the types. On the other hand, small probe size might prevent hybridisation occurring at all in a situation where strain variations were common. In the current study, an oligonucleotide of 30 bases was selected in an attempt to balance these opposing factors and, by searching the GenBank database it appeared that the sequences chosen from HPV-1a, HPV-6b and HPV-16 were different from any other PVs of known sequence. However, it was realised that some cross-reaction between HPV-6b and HPV-11 might occur since these types differed in only 4 bases with a run of 19 perfectly matched consecutive nucleotides (see

Fig 9) and this was found to be the case (see Specimens 7 and 22, Table 35).

The effectiveness of synthetic oligonucleotide probes does assume the accuracy of the sequence date lodged to date, and for HPV-16 at least, it has recently been shown that a single base was omitted from position 3906 in the original sequencing (Bubb et al., 1988) and indeed, it has been suggested that errors may be introduced from the bacterial strains used in cloning (Kasher and Roman, 1988). The advantages and disadvantages of short oligonucleotide probes are summarised in Table 37.

Selecting the best oligonucleotide sequences to give type specific HPV probes was difficult. Sequenced HPV types show a great degree of homology in the L1 region, but much less so in the L2 region, and it might have been possible to select sequences from within L2. However, the E6/E7 region, being important in transformation, was selected in the hope that strain differences between types of similar oncogenic involvement would be minimised in this region.

Apart from the report by Jablonski et al. (1986) with alkaline phosphatase labelled oligoprobes, all publications to date reporting the detection of specific viral sequences used ^{32}P labelling systems, either with polynucleotide kinase (Robertson et al., 1987; Sano et al., 1988) or terminal transferase (Lin et al., 1987). The latter enzyme was suggested in 1983 by Leary et al. to be a useful alternative to kinase labelling for biotinylated probes, but no results were included demonstrating its use. Terminal transferase has the advantage of being a one-step reaction and is less expensive in the amount of label required, but more than one biotinylated nucleotide may be added to the 3' end (Roychoudhury et al., 1976). Provided this did not create a probe with steric hindrance nor result in many unlabelled molecules, such addition might be advantageous and increase the sensitivity of the probe. It was therefore selected for labelling of the oligonucleotides used in this project. Current synthesising methods now allow the

Table 37 **Advantages and Disadvantages of Short Oligonucleotides in Hybridisation Studies (adapted from Coghlan et al., 1985)**

Advantages	Disadvantages (NB Most relate to "newness" of method)
1. They can be synthesised quickly.	1. Fewer labels have been tried.
2. They are easier for an ordinary lab to have prepared and to label.	2. The stability of probes has yet to be verified.
3. They give better overall tissue labelling since unlimited amount of probe are available.	3. Optimal conditions for short probes are not yet established.
4. They are consistent and have a higher specific activity.	4. Sequencing errors in published sequences lead to "wild goose chases".
5. Pure synthetic probes give a a lower background.	5. Cross-species hybridisation is more likely to be successful with longer probes.
6. Absence of poly(T) tails eliminates one source of background.	6. Some oligoprobes may fail because the region chosen is inaccessible.
7. Discriminating sequences for similar genes can be made.	
8. Known sequences may be altered for different species using preferred codons.	
9. Shorter probes may be more accessible to cellular mRNA.	
10. Probes may be synthesised from amino-acid sequence when DNA sequence unknown.	
11. Bacterial sequences in cloned probes are not a problem.	

addition of one biotin molecule to the 5' end of each oligonucleotide molecule during the synthetic process using an animo-linker. (Agrawal et al., 1986; Dr. Tom Brown, personal communication). Although HPV-6b and HPV-16 have been synthesised in this way, no results are available as to their effectiveness (Dr. Paul Kelly, Mercia Diagnostics Ltd., personal communication). Possibly one biotin in 30 nucleotides capable of binding to one copy of viral sequence may be less sensitive than required. Proportionately, this is similar to the level of substitution of thymidine by biotin-11-dUTP in a nick translation reaction (Kozma and Adinolfi, 1987), but with the longer probe, several biotin molecules will be present in each molecule of probe hybridised to a segment of viral genome. Terminal transferase labelling may therefore be preferable to labelling during the synthetic process.

Three ISH techniques were used in this study : biotinylated cloned probes of HPV-1a, HPV-11 and HPV-16 detected with streptavidin, biotinylated alkaline phosphatase and NBT/BCIP substrate (Detection Method 1) and oligonucleotide probes of HPV-1a, HPV-6b and HPV-16 detected with Detection Method 1 and with antibiotin, gold conjugated second antibody and silver enhancement (Detection Method 2). All were successful on formalin fixed sections of skin warts, though it was notable that sections of a simple plantar wart mounted on untreated slides were less likely to be lost with the shorter processing and hybridisation schedule for oligoprobes. In the small sample of tissues examined, good agreement was obtained between hybridisation results using cloned and oligo probes for HPV (Table 35). However, an apparent increase in sensitivity was achieved using HPV-16 E6 oligoprobe and Detection Method 2 (Table 36). It is appreciated that the same specimens were not tested by all three methods, but the saving in time by the use of oligoprobes, allowing completion of the process within one working day, coupled with increased sensitivity and a further saving in time during detection with the anti-biotin IGSS system, makes this a useful addition to ISH protocols. It would be

interesting to investigate normal, dysplastic and carcinomatous tissue from the uterine cervix using this method.

New labelling and detection systems are however constantly being tried, and it is probable that greater sensitivity and efficiency can yet be obtained. Urdea et al., (1988) recently reported for example that enzyme-modified oligonucleotides could be made to give sensitivities comparable to that of ^{32}P labelled probes.

While the role of HPV in cervical carcinogenesis is still in question it is important to use the most sensitive, practical and applicable assay available to assess the biological significance of its detection. The high degree of spatial resolution achieved with ISH makes it particularly useful when infection is clustered in small foci with a high copy number per cell as is more likely in early lesions. The use of ISH may therefore help to identify patients potentially at risk of malignant progression in the presence of HPV types which may integrate more readily. Furthermore, using single-stranded RNA probes from plasmids containing a phage SP6 promoter not only are stronger signals obtained than for DNA in the same tissues, but the interplay of virus replication and gene expression, cellular differentiation and transformation can be investigated (Stoler and Broker, 1986; Crum et al., 1988 a and b). The new dimension in diagnosis provided by ISH with quick-acting, non-radioactively labelled probes detected sensitively is worth exploiting further, although it is appreciated that such probes may fail to detect low copy number.

No attempt was made to assess the prevalence of HPV-DNA in a given population. Rather the aim of this part of the study was to perfect a rapid ISH technique which could be used in conjunction with immunocytochemical staining to give a better picture of the host response to HPV. This latter aspect did not progress as far as was envisaged because of technical difficulties. Whilst ISH worked well on paraffin sections, less success was achieved with frozen sections. On the other hand, many monoclonal antibodies work only on frozen

sections. This is particularly true for T cell markers. A recent report (Mason et al., 1988) suggests however that a new range of monoclonals suitable for phenotypic analysis of leucocytes in routine histological samples is becoming available. An alternative for LC is to use anti S-100 antiserum which detects some, but not all LC in formalin fixed tissue (Tay et al., 1987a) and this is being tried in conjunction with ISH using biotinylated probes (Dr. Kathryn McLaren, personal communication) on sections of HPV tumours from renal transplant patients. Of the many reports of HPV-ISH in the literature, while many mention the use of frozen sections in passing, few give details of results on them. In one series, however, frozen sections of mucosal papillomatous lesions were successfully detected using biotinylated cloned probes (Bouvard et al., 1986). Using their method, however, I was unable to retain the integrity of the sections, possibly due to the more severe denaturation stage of processing using boiling PBS.

HPV-DNA typing of cervical lesions can now be achieved with five different types of technique. Viral DNA can be extracted from a biopsy sample and spotted onto nitrocellulose or nylon filters (dot blots) or separated after restriction endonuclease digestion by gel electrophoresis before blotting (Southern blots; Southern, 1975). This gives a more specific reaction than dot blotting, gives information on the state of the viral genome and is the most sensitive method when there is a low copy number of viral genomes per cell, as in cervical tumours. It is the "gold standard" of DNA hybridisation techniques against which other methods should be measured, but it is expensive, time-consuming and less suitable when only a few cells in a sample are infected. Without preliminary extraction, viral DNA can be detected and typed by ISH as described above on tissue sections or by FISH (filter-ISH) (Wagner et al., 1984; Schneider et al., 1985), when exfoliated cells are immobilised on a membrane prior to hybridisation. As with dot-blotting, FISH is less specific and great care must be taken to avoid cross-hybridisation

(Wickenden et al., 1987).

A more recent DNA detection method involving amplification of target sequences through repeated cycles of denaturation and primer extension with DNA polymerase has been developed (Saiki et al., 1985). Polymerase chain reaction (PCR) was first applied to the search for HPV sequences by Shibata et al. (1988) in paraffin embedded tissue. Dewaxed tissue pellets were subjected to 40 cycles of amplification using oligomer primers and a ^{32}P end-labelled probe. HPV-16 sequences were successfully demonstrated in SiHa cells (1-2 copies/cell) and HPV-18 sequences in HeLa cells (10-50 copies/cell) and in several cervical and penile carcinomas. Dallas et al. (1989) report the use of PCR on pelleted cells from cervical scrapes using a probe directly labelled with alkaline phosphatase to give a very rapid detection method. Because the method is "exquisitely sensitive" great care must be taken to prevent non-specific positive results. Nevertheless, the use of PCR supports the suggestion that the prevalence of HPV infection is much greater than thought, with Young et al. (1989) finding HPV-11 or 16 in 7/10 women with no cytological abnormality and in all 38 women with cytological abnormalities. Indeed, Tidy et al., (1989) very recently reported 84% of 140 normal cervical samples, 67% of dyskaryotic smears and 100% of cervical carcinomas to be carrying HPV-16 sequences. This extremely high rate of infection in the general population begs the question of an aetiological role for HPV-16 in cervical neoplasia. The prevalence of multiple virus infections in normal and abnormal cervices using PCR (Bevan et al., 1989) supports the need for caution in interpretation.

5. Mouse Model

One of the major obstacles to HPV research remains the inability to produce sufficient amounts of infectious virus in the

laboratory. The system described by Kreider et al. (1986; 1987) in which human foreskin is infected with HPV-11 and grafted under the renal capsule gives experimental lesions similar to naturally occurring lesions and from which infectious virus can be recovered. Implanting under the renal capsule of a very small mouse is technically difficult and practice was gained using cervical biopsies, none of which remained as viable tissue. Attempts to reproduce the system using human foreskin and the small amount of mouse grown HPV-11 provided by Dr. Kreider met with limited success. Three of the four implants in the first experiment produced cysts, two of which were distinctly papillomatous and koilocytic and contained small numbers of HPV particles. None of the four mice implanted in the second experiment produced cysts and it seems likely that the human foreskin tissue used on this occasion was less viable or had more subcutaneous tissue preventing adsorption of the virus to basal cells.

A success rate of 25% (2 out of 8 attempts) shows that although possible, the system is difficult. Furthermore, Kreider noted that in his hands, the cysts took 3-5 months to develop. Given the short life expectancy of nude mice, the technical problems associated with this method are considerable. Recent work suggests that dexamethasone treatment of the mice might allow the development of experimental lesions from tissue implanted subcutaneously (Dr. J.W. Kreider, personal communication), a method technically much simpler than grafts under the renal capsule. It remains to be seen, however, whether the method can be adopted for larger scale production of infectious virus, or adapted for other HPV types.

6. The Multifactorial Process of Tumour Development

6.1 Initiating Events

There seems little doubt that the development of malignant

tumours of the cervix and other genital tissues is a multifactorial process in which HPV may play some role. This role could either be as an "initiator" or a "promoter", in a two-stage process, as first postulated by Berenblum (1941) for squamous cell carcinogenesis.

In cottontail rabbits, infection with CRPV represents the initial event to which the adapted host responds and suppresses tumour progression, but in the non-adapted domestic rabbit, suppression is less active and the continued expression of some viral functions allows for the development and maintenance of a malignant phenotype. The role of initiator has also been proposed for BPV-4 in the development of bovine alimentary canal carcinomas (Smith and Campo, 1988) although no viral DNA is expressed or even detected in the tumours themselves. These two extremes may reflect different roles for different PVs in malignant progression. In human cervical carcinomas, while the DNA of HPV of restricted types is found in the majority of cases, tumours which lack viral DNA, despite sensitive detection systems, may truly have lost the HPV genome. Similarly, although viral expression of restricted parts of the genome is usually found, this is not always so, (Lehn et al., 1985; Pater and Pater, 1988). However, if progression to tumorigenicity takes place through a series of steps only one of which is virus dependent, the persistence of the viral DNA or its loss at later stages may be unimportant and have little effect on the cell phenotype (Crawford 1986).

It has been suggested that in human genital carcinomas, the initiating role is played by another virus, herpes simplex virus (HSV) acting in a "hit and run" manner (zur Hausen, 1980; Galloway and McDougall, 1983). For several years, HSV itself was implicated in the development of cervical neoplasia (Rawls et al., 1973), but the evidence was mainly serological and inconclusive. Early studies had failed to detect HSV-2 nucleic acid in cervical tissue biopsies (zur Hausen et al., 1974) and even when assays became more sensitive, HSV-specific RNA could only be detected in 35-67% of tumours (McDougall et al., 1980;

Eglin et al., 1981; Maitland et al., 1981). Furthermore, no single sequence was always expressed in the HSV-positive specimens, and the proteins produced appeared to be so different that it seemed likely that they only gave some growth advantage rather than being necessary for the initiation or the maintenance of the transformed state (Galloway and McDougall, 1983). In addition, the higher incidence of HSV-2 antibodies in cases and controls could be explained by covariance, with the same factors predisposing to HSV-2 infections as to cervical carcinoma development. On the other hand, the ability of HSV to induce chromosomal breaks (Hampar and Ellison, 1961) and to induce cellular DNA repair (Nishiyama and Rapp, 1981) may be far more important than the presence of HSV-specific nucleic acids or protein. Recurrent herpetic infections could then lead to an accumulation of possible initiating events and increase the risk of malignant conversion in cervical papilloma infections (zur Hausen, 1980; 1982). The simultaneous presence of HSV and HPV in 4/13 cases of CIN and 4/30 cases of cervical cancer was recently shown (Di Luca et al., 1987), but a further 8 cases of CIN and 12 invasive tumours had detectable evidence only of HPV. Using PCR, Bevan et al., (1989) recently reported HSV or EBV with HPV in 19/36 abnormal smears, although HPV was present in all 36.

6.2 Risk Factors

In the promotion of the potentially tumorigenic cell, several risk factors have to be considered. Given the prevalence of HPV-16 in various countries in which it has been studied and the incidence now of cervical carcinoma, Pfister (1987b) calculated that the risk of HPV-16 infection in women was 10% and the risk of cervical cancer 1:300 of those infected. For HPV-6/11, the risk was 1:2000-3000 of those infected, which still exceeds the risk for other human tumour viruses such as human T-cell lymphotrophic virus, type 1 (HTLV-1), Epstein-Barr virus (EBV) and hepatitis B virus (HBV). While experimental data

supports an oncogenic potential for HPV, epidemiological evidence is still limited and is based on studies which do not satisfy basic epidemiological requirements of sample size, careful unbiased selection of cases and matching controls, and detection systems of comparable specificity and sensitivity (Munoz et al., 1988). Indeed, doubt is cast on the relevance of HPV in cervical cancer by the high prevalence of HPV-16/18 in normal cervical cells and tissues (Cox et al., 1986; Macnab et al., 1986; De Villiers et al., 1987; Meanwell et al., 1987; Reeves et al., 1987; Schneider et al., 1987; Murdoch et al., 1988). However, if other factors act synergistically, HPV infection would still represent an important stage in the development of cervical carcinoma.

Heavy and prolonged smoking has been known for decades to be a major risk factor in the development of malignancies of the respiratory tract and implicated also in cervical cancer (Winkelstein, 1977; Clarke et al., 1982) with Trevathan and colleagues (1983) concluding that the cumulative exposure was most important, with the risk being greatest in those who began smoking in their teenage years (La Vecchia et al., 1986). Certainly, carcinogenic nicotine products have been shown to accumulate in vaginal fluids (Sasson et al., 1985). It has also been shown that smoking upsets vitamin A metabolism (Yamasaki et al., 1977), and since vitamin A in high doses can have beneficial effects on some warts (Blanchet-Bardon and Lutzner, 1985), its reduction may aid progression. Furthermore, smoking can lead to suppression of immune responses by exerting an anti-oestrogenic effect (Michnovicz et al., 1986) and by reducing LC in cervical epithelium (Barton et al., 1988). This could lead to the reactivation of viruses latent in cervical tissue (Grail and Norval, 1988).

It is very difficult to separate correlated factors and various aspects of social behaviour have been considered with conflicting results as to their individual relevance. High sexual activity is generally considered to be a major risk factor, and in breaking this down to age at first intercourse, multiple sexual partners and pregnancy outside

marriage, Harris and colleagues (1980) noted that these risk factors were identical for cervical dysplasias of all grades and for invasive cervical carcinoma. While the number of sexual partners exerted its effects independently of age at first intercourse, the reverse was not found. This study therefore failed to support the suggestion that adolescence was the period when the cervix was most vulnerable to the effects of sexual behaviour. Recently, in a study of the development of CIN in Italian girls under 20, the major risk factors were shown to be the number of sexual partners and the existence of genital warts (Zaninetti et al., 1986). No association with smoking was found in this study, presumably due to the short duration of the smoking habit.

The greatest single risk factor would appear to be the number of sexual partners a female or her single male partner has encountered (McCance, 1986). Skegg and colleagues (1982) showed that in societies where male promiscuity was high and female promiscuity low, the incidence of cervical cancer is high. This was recently confirmed in a group of poor Hispanic women : cervical cancer patients were more than five times as likely as controls to be married to husbands who had had more than 20 sexual partners (Zunzunegui et al., 1986). By considering different cultural and religious practices, an additive effect of age at first coitus and the level of penile hygiene was noted by Jayant (1987).

The use of oral contraceptive pills (OCPs) has also been implicated as a risk factor in the progression of cervical dysplasias to cancer. In two out of three studies, the risk of dysplasia appeared to increase with increased duration of OCP use, but the confounding influences of sexual behavious patterns were not assessed (Prentice and Thomas, 1987). In three studies on the effect of OCP use on invasive cervical cancer, a risk was shown even when controlled for sexual variables, as in the study by Vessey et al. (1983) where nearly 10,000 women attending family planning clinics in Britain were followed for 10 years. Thirteen cases of cervical carcinoma developed in OCP users

with more than 6 years useage, while no cases in matched controls using intra uteric devices were found. However, in this study, the confounding variable of smoking was not assessed (Prentice and Thomas, 1987).

6.3 Progression

In a gradually evolving hypothesis, zur Hausen has proposed that cancers develop as a result of failing intracellular control of persisting viral genomes in proliferating cells (zur Hausen, 1980; 1982; 1986). In HPV infection of cervical epithelium, a cellular interfering factor exists which, in the lower layers of the epidermis, suppresses early viral functions, but is itself switched off by differentiation allowing viral DNA replication to ensue. An earlier switch-off could result from modification of cellular genes as a result of smoking, or infections with initiating properties such as HSV, CMV or chlamydia and/or the accumulation of mutagenic metabolites from chronic inflammation, all of which would have more disastrous effects on the hormonally controlled cells of the cervical transformation zone than on external genitalia. This would explain the higher risk of cervical than of vulvar or penile cancers and also the older age of onset of the latter types. The presence of such a cellular factor regulating HPV expression has been suggested by the finding that, while HeLa cells containing integrated HPV-18 form tumours in nude mice, hybrids of HeLa and normal human fibroblasts or keratinocytes retain their HeLa characteristics in vitro but fail to produce tumours in nude mice (Schwarz et al., 1987; Rosl et al., 1988).

HPV-16/18 could further promote tumour development by their ability to integrate into the host genome, usually with integration occurring within E1/E2 and transcription of E6/E7 continuing. It was noted, however, that the message from the E6 region of HPV-18 integrated genomes was altered by the insertion of cell nucelotides (Schneider-Gadicke and Schwarz, 1986) to cause a frame shift which could result in the expression of a protein distantly resembling

epidermal growth factor. Interestingly, HPV-6/11 lack the appropriate splice site and this might explain their lower tumorigenic potential. Nevertheless, integration of HPV-11 can occasionally occur (Byrne et al., 1987), transcription of HPV-16/18 does not always occur (Lehn et al., 1985; Pater and Pater, 1988) and progression of HPV-6/11 lesions can be as common as the progression seen in HPV-16/18 lesions (Syrjanen et al., 1988). The insertion site of an integrated viral genome may be more relevant with activation of nearby cellular oncogenes (Riou et al., 1985; Matlashewski et al., 1987) being the real trigger of oncongenic progression. It could even be that excision of an integrated viral DNA sequence with a neighbouring cellular control region are the events which finally precipitate conversion to a carcinogenic cell (Crawford, 1986). In this case, all evidence of virus infection would be lost.

If transformation to malignancy is a series of steps only one of which is virus dependent, the persistence of the genome at later stages is unnecessary. Yet to play a significant role, the presence of HPV at some stage must always occur. Only by years of follow-up of large numbers of women on a regular basis could there be a chance of proving epidemiologically an aetiological role for HPV in cervical dysplasia. Such a study would be lengthy, expensive and dependent on adequate storage facilities for large number of sample (Munoz et al., 1988), but these criteria are being currently met in a large London study involving the collection of 100,000 cervical scrapes with retrieval of those from patients developing CIN and matched controls who do not (Professor A.B. Malcolm, personal communication). The use of sensitive detection methods such as PCR on these samples (Tidy et al., 1989) will yield valuable and hopefully conclusive results.

7. Concluding Comments

Few studies of the involvement of HPV in cervical dysplasia take into account the host response to the infection. In the preceding section, various risk factors are considered, but allowances are not made for differing host responses to these factors. The level of nicotine metabolites required to exert an immunosuppressive effect will vary from individual to individual, the development of chronic inflammation in response to exposure to foreign antigens may be more pronounced in some than in others, the effect of OCP use will be dependent on the hormonal balance already existing in the individual and the response to HPV will be dependent on the state of the epidermis at the time of infection. It becomes difficult to predict the outcome of such complex encounters.

The current study was initiated in an attempt to find markers of host response which would indicate the relevance of HPV infection in cervical dysplasia and was commenced at a time when HPV in normal tissues had not yet been detected. It became evident that finding the best HPV antigens to assess in vitro assays was in itself a major hurdle. Disrupted virions revealing group specific antigens will still detect the most commonly acquired HPV infections whatever their type and the inability to produce specific HPV types readily in cell culture is as big a problem as twenty years ago. Great promise for the production of natural HPV antigens lies in the work of McCance et al. (1988) with "raft cultures" on which differentiating keratinocytes can support HPV replication, or from the nude mouse work of Kreider et al. (1986; 1987) if the infection and implantation method can be adapted to HPV types other than HPV-11. The studies reported here showed that Kreider's work could be reproduced, although the efficiency of the technique was low and the complexity of operation high. Certainly, naturally produced antigens are likely to yield more constructive and meaningful results than the two systems currently available of fusion proteins and

synthetic peptides. Fusion proteins were tried here in LPA with some success, but the problems of removal of contaminating bacterial sequences and of alteration to the specified HPV proteins by bacterial passage are considerable, while the disadvantage of synthetic peptides must lie in their conformational arrangements.

Even with the most suitable antigenic preparations, however, it is questionable whether examination of the systemic response, either humoral or cellular, to what is normally a locally contained infection, would now be considered the most valuable approach. Attempts to use in vitro assays of systemic responsiveness can be made more relevant, for example, by the use in LPA of APC from epithelium of the same type or area as that of the lesion. However, such studies can probably be compared to looking for "a needle in a haystack" and future work would do better to concentrate on local responses.

Nevertheless, full examination of the local responses in a human system is difficult using functional assays, and information is currently being gathered from simpler and more adaptable cytochemical studies. In the last three years, increasing interest has been shown in lymphocyte markers within the area of HPV infection, but none have looked adequately at the HPV itself in the same lesion. Histological criteria such as the presence of koilocytes have been used to denote HPV infection, but a combination of HPV-typing by DNA hybridisation or of gene transcription by RNA hybridisation in combination with markers of immune responsiveness would be more relevant.

The technical difficulties in obtaining sensitive ISH without radiolabelling of probes were evident in the current study and the time involved in the successful development of a new tool in ISH using biotinylated synthetic oligonucleotides, effectively curtailed studies involving multiple markers. Synthetic oligonucleotides themselves offer exciting opportunities for the rapid diagnosis of viral disease in situations where antigen detection is inappropriate, and I am already applying this approach to the search for respiratory viruses in

nasopharyngeal secretions from children. The investigation of multiple markers in cervical dysplasia is, however, an area well worth more comprehensive study and should involve the examination not only of clinically and histologicaly abnormal material, but of apparently normal tissues from the same patient and apparently normal tissues from clinically and histologically normal people. As more monoclonal antibodies suitable for use on paraffin sections become available, these techniques will be increasingly feasible on archival material to supplement the data obtained from controlled studies.

In the search for models of PV cancinogenesis, animal models such as Mastomys minutus or the papillomatous lesions of hairless mice may prove to be of increasing value, and the CRPV model could be further exploited in the investigation of cellular actions and interactions. Furthermore, for HPV itself, the actions of cutaneous HPVs should not be forgotten, and it could be that immunocompromised people will increasingly provide living models in whom the host response can be investigated. Much has still to be done in analysing the part played by HPV-5 and HPV-8 in malignant and premalignant lesions in immunocompromised patients and relating it to the normal state of the skin in healthy individuals.

At last more researchers are directing their attention to unravelling the complex and enigmatic interactions of the pathogenesis and host response to HPV. In the meantime, experimental molecular data on HPV have been accumulating very rapidly and controlled epidemiological data on the involvement of HPV in the progression of dysplasias will be available for analysis. The next ten years of HPV research should be exciting.

ACKNOWLEDGEMENTS

I would firstly like to thank my supervisor, Dr. Mary Norval for much helpful discussion and criticism. The support of all those in the Virus Research Laboratory, especially Mrs. Jean Maingay, Dr. Sarah Howie and Mr. Bill Neill, was much appreciated. The unflagging help of our research nurse, Mrs. Muriel Murray, in obtaining specimens and patient information was invaluable and I gratefully acknowledge her contribution. My thanks are due also to all those in the Department of Bacteriology who gave me help at one time or another, and for the range of facilities provided.

I am most grateful to Dr. George Smart, Department of Gynaecology for his continued interest and permission to study his patients, and to Dr. Stewart Fletcher, Department of Pathology, for help with histological interpretation and access to histological data. Many friends and colleagues in the Departments of Dermatology, Genito-urinary Medicine, Gynaecology and Pathology have given help and shown their support, individually and through the Edinburgh Papilloma Virus Group and I am grateful to them. I would particularly like to thank Dr. Mary Bunney for her example in perseverence with the study of warts, and for the photographs comprising Figure 4; Dr. Claire Benton for providing specimens from patients with recalcitrant warts; Dr. Stewart Fletcher and Dr. Gemma Rebello for the photographs reproduced in Figure 5; Mr. Paul Bishop for sharing some of his ISH slides and results with me. I would also like to thank my daughter Alison for the adapted drawings of Figure 6. I am grateful to Dr. Lionel Crawford, Dr. Lawrence Banks and Dr. John Doorbar for the provision of fusion proteins, and to Dr. Tom Brown, Department of Chemistry, for synthesising the required oligonucleotides.

The readiness of Dr. Hamish Inglis, Director of the Regional Virus Laboratory at the City Hospital to allow me to work flexible hours

in that laboratory so that this project could be carried out, was a great help and much appreciated. Care of the nude mice at the City Hospital was provided by Mrs. Joan Barnett, Miss Tracey Kilpatrick and Mrs. Sandra Hay, and Dr. Rod Else, Department of Veterinary Pathology helped with the mouse histology. The financial assistance of the Cancer Research Campaign for the duration of this project is acknowledged, and I am indebted to Lothian Health Board and the CRC for major contributions towards attending the 4th International Papilloma Workshop in Kuopio in 1985 and the 6th Workshop in Washington in 1987.

I am most grateful to Mrs. Ann MacInnes for the innumerable hours spent typing and correcting this thesis, and to Mrs. Marilyn Cole for the painstaking way in which she reproduced my complex tables and reference list. I would like to thank Professor Gerry Collee, Dr. Marie Ogilvie and my mother, Mrs. Freida Muir for careful reading of the manuscript.

Above all, however, I am indebted to all of my family - to my husband Andrew who has been incredibly supportive, patient and understanding over the last four years, and to my children Alison, Douglas and Catherine who have put up with a great deal of inconvenience and loss of family time so that I might achieve this goal.

REFERENCES

Aberer W, Schuler G, Stingl G, Honigsmann H and Wolff K (1981). Ultraviolet light depletes surface markers of Langerhans cells. J invest Dermatol 76: 202-210.

Agrawal S, Christodoulou C and Gait MJ (1986). Efficient methods for attaching non-radioactive labels to the 5' ends of synthetic oligodeoxynucleotides. Nucleic Acid Res 14: 6227-6245.

Aiba S and Tagami H (1984). HLA-DR antigen expression on the keratinocyte surface in dermatoses characterised by lymphocytic exocytosis (e.g. pityriasis rosea). Br J Dermatol 111: 285-294.

Alloub MI, Barr BBB, McLaren KM, Smith IW, Bunney MH and Smart GE (1989). Human papillomavirus infection and cervical intraepithelial neoplasia in women with renal allografts. Br Med J 298: 153-156.

Amtmann E, Randeria J and Wayss K (1987). Interaction of papillomaviruses with carcinogens and tumor promoters. In Papillomaviruses (Ed Steinberg BM. Bransdma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 259-266.

Amtmann E, Volm M and Wayss K (1984). Tumour induction in the rodent Mastamys natalensis by activation of endogenous papilloma virus genomes. Nature 308: 291-292.

Androphy EJ, Dvoretzky I, Maluish AE, Wallace HJ and Lowy DR (1984). Response of warts in epidermodysplasia verruciformis to treatment with systemic and intralesional alpha interferon. J Am Acad Dermatol 11: 197-202.

Androphy E, Schiller JT and Lowy DR (1985). Identification of the protein encoded by the E6 transforming gene of bovine papillomavirus. Science 230: 442-445.

Aubock J, Romani N, Grubauer G and Fritsch P (1986). HLA-DR expression on keratinocytes is a common feature of diseased skin. Br J Dermatol 114: 465-472.

Avgerinou G, Georgala S, Theodoridis A, Vareltzdis A and Stratigos J (1986). Reduction of cell mediated immunity in patients with genital warts of long duration. Genitourin Med 62: 396-398.

Bagot M, Heslaw M, Roujeau JC, Lebon P and Levy JP (1985). Human epidermal cells are more potent than peripheral blood mononuclear cell for the detection of weak allogeneic or virus specific primary responses in vitro. Cell Immunol 94: 215-224.

Baird PJ (1983). Serological evidence for the association of papillomavirus and cervical neoplasia. Lancet ii: 17-18.

Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA and Howley PM (1987). Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol 61: 962-971.

Banks L, Spence P, Androphy E, Hubbert N, Matlashewski G, Murray A and Crawford L (1987). Identification of human papillomavirus type 18 E6 polypeptide in cells derived from human cervical carcinomas. J Gen Virol 68: 1351-1359.

Barr BBB, Benton EC, McLaren K, Bunney MH, Smith IW, Blessing K and Hunter JAA (1989). Human papillomavirus infection and skin cancer in renal allograft recipients. Lancet i: 124-128.

Barton SE, Maddox PH, Jenkins D, Edwards R, Chzick J and Singer A (1988). Effect of cigarette smoking on cervical epithelial immunity: a mechanism for neoplastic change? Lancet ii: 652-654.

Batchelor JR, Lombardi G and Lechler RI (1989). Speculations on the specificity of suppression. Immunol Today 10: 37-40.

Becker J, Behem J, Loning TH, Reichart P and Geerlings H (1985). Quantitative analysis of immunocompetent cells in human normal oral and uterine cervical mucosa, oral papillomas and leukoplakias. Arch Oral Biol 30: 257-264.

Beckmann AM, Myerson D, Daling JR, Kiviat NB, Fenoglio CM and McDougall JK (1985). Detection and localisation of human papillomavirus DNA in human genital condylomas by in situ hybridisation with biotinylated probes. J Med Virol 16: 265-273.

Bedell MA, Jones KH, Grossman SR and Laimins LA (1989). Identification of human papillomavirus type 18 transforming genes in immortalised and primary cells. J Virol 63: 1247-1255.

Bedell MA, Jones KH and Laimins LA (1987). The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and RAT-1 cells. J Virol 61: 3635-3640.

Beiss BK, Sundberg JP, Douglas JM, Burk RD, Ritter DB and Kadish AS (1987). Host immune responses to genital and laryngeal human papillomavirus infections. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 387-392.

Belsito DV, Sanchez MR, Baer RL, Valentine F and Thorbecke J (1984). Reduced Langerhans' cell Ia antigen and ATPase activity in patients with the acquired immunodeficiency syndrome. New Eng J Med 310: 1279-1282.

Bennett WM and Norman DJ (1986). Action and cytotoxicity of cyclosporine. Ann Rev Med 37: 215-224.

Berenblum I (1941). The mechanism of carcinogenesis. A study of the significance of cocarcinogenic action and related phenomenon. Cancer Res 1: 807-814.

Berman A and Berman JE (1978). Efflorescence of new warts: A sign of onset of involution in flat warts. Br J Dermatol 99: 179-182.

Berman A and Winkelmann RK (1977). Flat warts undergoing involution. Arch Dermatol 113: 1219-1221.

Berman A and Winkelmann RK (1980). Involuting common warts. J Am Acad Dermatol 3: 356-362.

Berman B, Duncan MR, Smith B, Ziboh VA and Palladino M (1985). Interferon enhancement of HLA-DR antigen expression on epidermal Langerhans cells. J Invest Dermatol 84: 54-58.

Bevan IS, Blomfield PI, Johnson MA, Woodman CBJ and Young LS (1989). Oncogenic viruses and cervical cancer. Lancet i: 907-908.

Black PH, Hartley JN, Rowe WP and Huebner RJ (1963). Transformation of bovine tissue culture cells by bovine papilloma virus. Nature 199: 1016-1018.

Blanchet-Bardon C and Lutzner MA (1985). Interferon and retinoid treatment of warts. Clin Dermatol 3: 195-199.

Booth JC, Harrington G, Aziz TA and Stern H (1979). Comparison of enzyme-linked immunosorbent assay (ELISA) technique and complement fixation test for estimation of cytomegalovirus IgG antibody. J Clin Pathol 32: 122-127.

Bos SD and Kapsenberg ML (1986). The skin immune system: its cellular constituents and their interactions. Immunol Today 7: 235-240.

Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W and zur Hausen H (1984). A new type of papillomavirus DNA and its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J 3: 1151-1157.

Boshart H and zur Hausen H (1986). Human papillomaviruses in Buschke-Lowenstein tumours: physical state of the DNA and indentification of a tandem duplication in the non-coding region of human papillomavirus 6 subtype. J Virol 58: 963-966.

Bouvard V, Chardonnet Y, Viac J and Thivolet J (1986). Use of molecular in vitro hybridisation technique in human papillomavirus pathology. Microbios Lett 32: 147-156

Boxer RJ and Skinner DG (1977). Condylomata acuminata and squamous cell carcinoma. Urology 9: 72-78.

Braathen LR and Thorsby E (1983). Human epidermal Langerhans cells are more potent than blood monocytes in inducing some antigen-specific T-cell responses. Br J Dermatol 108: 139-146.

Brackmann KH, Green M, Wold WSM, Rankin A, Loewenstein PM, Cartas MA, Sanders PR, Olson K, Orth G, Jablonska S, Kremsdorf D and Favre M (1983). Introduction of cloned human papillomavirus genomes into mouse cells and expression at the RNA level. Virology 129: 12-24.

Brahic M and Haase A (1978). Detection of viral sequences of low reiteration frequency by in situ hybridisation. Proc Natl Acad Sci USA 75: 6125-6129.

Brandsma J, Abramson A, Sciubba J, Shah K, Barrezueta N and Galli R (1987). Papillomavirus infection of the nose. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 301-308.

Brandsma JL, Steinberg BM, Abramson AL and Winkler B (1986). Prescence of human papillomavirus type 16 related sequences in verrucous carcinoma of the larynx. Cancer Res 46: 2185-2188.

Braun L, Farmer ER and Shah KV (1983). Immunoperoxidase localisation of papillomavirus antigen in cutaneous warts and bowenoid papulosis. J Med Virol 12: 187-193.

Breedis C, Berhick L and Anderson TF (1962). Fractionation of Shope papillomavirus in caesium chloride density gradients. Virology 17: 84-94.

Breitburd F, Croissant O and Orth G (1987). Expression of human papillomavirus type-1 E4 gene products in warts. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 115-122.

Brigati DJ, Myerson D, Leary JJ, Spalholz B, Travis SZ, Fong CKY, Hsiung GD and Ward DC (1983). Detection of viral genomes in cultured cells and paraffin embedded tissue sections using biotin-labelled hybridisation probes. Virology 126: 32-50.

Broderson I, Genner J and Brodthagen H (1974). Tuberculin sensitivity in BCG-vaccinated children with common warts. Acta Dermatovenereol 54: 291-292.

Broker TR and Botchan M (1986). Papillomaviruses: retrospectives and prospectives. In DNA Tumour Viruses: Control of Gene Expression and Replication. (Ed. Botchan M, Grodzicker T and Sharp PA). Cancer Cells 4: Cold Spring Harbor (NY) Laboratory, 17-36.

Brown MD, Ellis CN, Billings J, Cooper KD, Baadsgaard O, Headington JT and Voorhees JJ (1988). Rapid occurrence of nodular cutaneous T-lymphocyte infiltrates with cyclosporin therapy. Arch Dermatol 124: 1097-1100.

Browne HM, Churcher MJ, Stanley MA, Smith GL and Minson AC (1988). Analysis of the L1 gene product of human papillomavirus type 16 by expression in a vaccinia virus recombinant. J Gen Virol 69: 1263-1273.

Bubb V, McCance DJ and Schlegel R (1988). DNA sequence of the HPV 16 E5 ORF and the structural conservation of its encoded protein. Virology 163: 243-246.

Bunney MH (1982). Viral Warts: Their Biology and Treatment. Oxford University Press.

Bunney MH, Nolan MW, Buxton PK, Going SM and Prescott RJ (1984). The treatment of resistant warts with intralesional bleomycin: a controlled clinical trial. Br J Dermatol 110: 197-207.

Burnett TS and Gallimore PH (1983). Establishment of a human keratinocyte cell line carrying complete human papillomavirus type 1 genomes: Lack of vegetative viral DNA synthesis upon keratinisation. J Gen Virol 64: 1509-1520.

Burnett TS and Gallimore PH (1985). Introduction of cloned human papillomavirus la DNA into rat fibroblasts: integration, de novo methylation and absence of cellular morphological transformation. J Gen Virol 66: 1063-1972.

Burns J, Graham AK, Frank C, Fleming KA, Evans MF and McGee JO'D (1987). Detection of low copy human papillomavirus DNA and mRNA in routine paraffin sections of cervix by non-isotypic in situ hybridisation. J Clin Pathol 40: 858-864.

Butel JS (1972). Studies with human papilloma virus modeled after known papovavirus systems. J Natl Cancer Inst 48: 285-299.

Byrne JC, Tsao M-S, Fraser RS and Howley PM (1987). Human papillomavirus-11 DNA in a patient with chronic laryngotracheobronchial papillomatosis and metastatic squamous-cell carcinoma of the lung. New Eng J Med 317: 873-878.

Campion MJ, Singer A, Clarkson PK and McCance DJ (1985). Increased risk of cervical neoplasia in consorts of men with penile condylomata acuminata. Lancet i: 943-946.

Campo MS and Coggins LW (1982). Molecular cloning of bovine papillomavirus genomes and comparison of their sequence homologies by heteroduplex mapping. J Gen Virol 63: 255-264.

Campo MS and Jarrett WFH (1986). Papillomavirus infection in cattle: viral and chemical co-factors in naturally occurring and experimentally induced tumours. In "Papillomaviruses": CIBA Foundation Symposium 120: 117-135.

Campo MS and Jarrett WFH (1987). Papillomaviruses and Disease. Soc Gen Microbiol Symp 40: 215-242.

Campo MS, Moar MH, Jarrett WFH and Laird HM (1980). A new papillomavirus associated with alimentary cancer in cattle. Nature 286: 180-182.

Campo MS, Moar MH, Sartirana ML, Kennedy IM and Jarrett WF (1985). The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. EMBO J 4: 1819-1825.

Campo MS and Spandidos DA (1983). Molecularly cloned bovine papillomavirus DNA transforms mouse fibroblasts in vitro. J Gen Virol 64: 549-557.

Carr MM, McVittie E, Guy K, Gawkrodger DJ and Hunter JAA (1986). MHC Class II antigen expression in normal human epidermis. Immunol 59: 223-227.

Chardonnet Y, Viac J and Thivolet J (1986). Langerhans cells in human warts. Br J Dermatol 115: 669-675.

Chardonnet Y, Viac J, Staquet MJ and Thivolet J (1985). Cell-mediated immunity to human papillomavirus. Clin Dermatol 3: 156-164.

Chen EY, Howley PM, Levinson AD and Seeburg PH (1982). The primary structure and genetic organisation of the bovine papillomavirus type 1 genome. Nature 299: 529-534.

Chorzelski T, Jarzabek-Chorzelska M, Jablonska S, Orth G and Rzesa G (1983). An immunofluorescence complement-fixation test for detection of human papillomavirus in various warts and wart-like lesions of epidermodysplasia verruciformis. Arch Dermatol Res 275: 53-57.

Chow LT, Hirochika H, Nasseri M, Stoler MH, Wolinsky SM, Chin MT, Hirochika R, Arvan DS and Broker TR (1987). Human papillomavirus gene expression. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 65-72.

Chretien JH, Esswein JG and Garagusi VF (1978). Decreased T cell levels in patients with warts. Arch Dermatol 114: 213-215.

Ciuffo G (1907). Innesto positivo con filtrado di verruca volgare. Giorno Ital Mol Venereol 48: 12-17.

Clarke EA, Morgan RW and Newman AM (1982). Smoking as a risk factor in cancer of the cervix: additional evidence from a case: control study. Am J Epidemiol 115: 59-66.

Clertant P and Seif I (1984). A common function for polyoma virus large-T and papillomavirus E1 proteins? Nature 311: 276-279.

Cocchia D, Michetti F and Donato R (1981). Immunochemical and immunocytochemical localisation of S-100 antigen in normal human skin. Nature 294: 85-87.

Coggin JR and zur Hausen H (1979). Workshop on Papillomaviruses and Cancer. Cancer Res 39: 545-546.

Cole ST and Danos O (1987). Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. J Mol Biol 193: 549-608.

Collins JE, Jenkins D and McCance DJ (1988). Detection of human papillomavirus DNA sequences by in situ DNA-DNA hybridisation in cervical intraepithelial neoplasia and invasive carcinoma: a retrospective study. J Clin Pathol 41: 289-295.

Cox MF, Meanwell CA, Maitland NJ, Blackledge G, Scully C and Jordan JA (1986). Human papillomavirus type-16 homologous DNA in normal human ectocervix. Lancet ii: 157-158.

Crawford LV (1969). Nucleic acids of tumour viruses. Adv Virus Res 14: 89-152.

Crawford L (1986). Criteria for establishing that a virus is oncogenic. In "Papillomaviruses": Ciba Foundation Symposium 120: 104-110.

Crawford LV and Crawford EM (1963). A comparative study of polyoma and papilloma viruses. Virology 21: 258-263.

Crawford LV and Waring MJ (1967). The supercoiling of papilloma-virus DNA. J Gen Virol 1: 387-390.

Cripe TP, Haugen TH, Turk JP, Tabata-Bai F, Schmid PG, Durst M, Gissmann L, Roman R and Turek LP (1987). Transcriptional regulation of the human papillomavirus 16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and regresses gene products: implications for cervical cercinogenesis. EMBO J 6: 3745-3753.

Croissant O, Breitburd F and Orth G (1985). Specificity of cytopathic effect of cutaneous human papillomaviruses. Clin Dermatol 3: 43-55.

Crook T, Morgenstern JP, Crawford L and Banks L (1989). Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-ras. EMBO J 8: 513-519.

Crum CP, Ikenberg H, Richart RM and Gissmann L (1984). Human papillomavirus type 16 and early cervical neoplasia. New Eng J Med 310: 880-889.

Crum CP, Nuovo G, Friedman D and Silverstein SJ (1988a). Accumulation of RNA homologous and human papillomavirus type 16 Open Reading Frames in genital precancers. J Virol 62: 84-90.

Crum CP, Nuovo G, Friedman D and Silverstein SJ (1988b). A comparison of biotin and isotype-labelled ribonucleic acid probes for in situ detection of HPV-16 ribonucleic acid in genital precancers. Lab Investigation 58: 354-359.

Cubie HA (1972a). Laboratory investigations in the study of human papilloma virus. M.Sc. Thesis, University of Edinburgh.

Cubie HA (1972b). Serological studies in a student population prone to infection with human papilloma virus. J Hyg Camb 70: 677-690.

Cubie HA (1974). Experiments with human papillomavirus in cell culture. Br J Dermatol 91: 569-571.

Cubie HA (1976). Failure to produce warts on human skin grafts on "nude" mice. Br J Dermatol 94: 659-665.

Dallas PB, Flanagan JL, Mightingale BN and Morris BJ (1989). Polymerase chain reaction for fast non-radioactive detection of high- and low-risk papillomavirus types in routine cervical specimens and in biopsies. J Med Virol 27: 105-111.

Dalmat HT (1958). Arthropod transmission of rabbit papillomatosis. J exp Med 108: 9-19.

Danos O, Engel LW, Chen EY, Yaniv M and Howley PM (1983). Comparative analysis of the human type 1a and bovine type 1 papillomavirus genome. J Virol 46: 557-566.

Danos O, Katinka M and Yaniv M (1982). Human papillomaviruses 1a complete DNA sequence: a novel type of genome organisation among Papovaviridae. EMBO J 1: 231-236.

Danos O, Mulligan RC and Yaniv M (1986). Production of spliced DNA copies of the cottontail rabbit papillomavirus genome in a retroviral vector. In "Papillomaviruses": CIBA Foundation Symposium 120: 68-82.

Dartman K, Schwarz E, Gissmann L and zur Hausen H (1986). The nucleotide sequence and genome organisation of human papillomavirus type 11. Virology 151: 124-130.

Daynes RA, Spangrude GJ, Roberts LK and Krueger GG (1985). Regulation by the skin of lymphoid cell recirculation and localisation properties. J Invest Dermatol 85: 14S-20S.

Delap R, Friedman-Kien A and Rush MG (1976). The absence of human papillomaviral DNA sequences in condylomata accuminata. Virology 74: 268-272.

Del Mistro A, Braunstein JD, Halwer M and Koss LG (1987). Identification of human papillomavirus types in male urethral condylomata accuminata by in situ hybridisation. Human Pathol 18: 936-940.

Devereux J, Haeberli P and Smithies O (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid Res 12: 387-395.

De Villiers EM, Gissmann L and zur Hausen H (1981). Molecular cloning of viral DNA from human genital warts. Virology 40: 932-935.

De Villiers EM, Weidauer H, Otto H and zur hausen H (1985). Papillomavirus DNA in human tongue carcinomas. Int J Cancer 36: 575-578.

Di Luca D,Rotola A, Pilotti S, Monini P, Caselli E, Rilke F and Cassai E (1987). Simultaneous presence of herpes simplex and human papillomavirus sequences in human genital tumours. Int J Cancer 40: 763-768.

Di Paulo JA, Pirisi L, Popescu NC, Yasumoto S and Doniger J (1987). Progressive changes induced in human and mouse cells by human papillomavirus type-16 DNA. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 253-257.

Dillner L, Bekassy Z, Jonsson N, Moreno-Lopex J and Blomberg J (1989). Detection of IgA antibodies against human papillomavirus in cervical secretions from patients with cervical intraepithelial neoplasia. Int J Cancer 43: 36-40.

Donnenberg AD, Elfenstein GJ and Santos GW (1984). Secondary immunisation with a protein antigen (Tetanus toxoid) in man; characterisation of tumor and cell-mediated regulatory events. Scand J. Immunol 20: 279-289.

Doorbar J, Campbell D, Grand RJA and Gallimore PH (1986). Identification of the human papillomavirus-1a E4 gene products. EMBO J 5: 355-362.

Doorbar J, Evans HS, Coneron I, Crawford LV and Gallimore PH (1988). Analysis of HPV-1 E4 gene expression using epitope defined antibodies. EMBO J 7: 825-833.

Doorbar J and Gallimore PH (1987). Identification of proteins encoded by the L2 and L2 open reading frames of human papillomavirus 1a. J Virol 61: 2793-2799.

Drijkoningen M, De Wolf-Peeters, De Greef H and Desmet V (1988). Epidermal Langerhans cells, dermal dendritic cells and keratinocytes in viral lesions of skin and mucous membranes: an immunohistochemical study. Arch Dermatol Res 280: 220-222.

Drummond JE, Shah KV and Donnenberg AD (1985). Cell mediated immune responses to BK virus in normal individuals. J Med Virol 17: 237-247.

Duff TB (1971). Laryngeal papillomatosis. J Laryngol Otol 85: 947-956.

Dunn ACG and Ogilvie MM (1968). Intranuclear virus particles in human genital wart tissue: observations on the ultrastructure of the epidermal layer. J Ulstrastruct Res 22: 282-295.

Durst M, Croce CM, Gissmann L, Schwartz E and Huebner K (1987). Papillomavirus sequences integrate near cellular oncogenes on some cervical carcinomas. Proc Natl Acad Sci USA 84: 1070-1074.

Durst M, Gissmann K, Ikenberg H and zur Hausen H (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci USA 80: 3812-3815.

Durst M, Kleinheinz A, Hotz M and Gissmann L (1985). The physical state of the human papillomavirus type 16 DNA in benign and malignant genital tumours. J Gen Virol 66: 1515-1522.

Dvoretzky I, Shober R, Chattopadhyay SK and Lowy DR (1980). A quantitative in vitro focus assay for bovine papilloma virus. Virology 103: 369-375.

Eglin RP, Sharp F, Maclean AB, Macnab JCM, Clements JB and Wilkie NM (1981). Detection of RNA complementary to herpes simplex virus DNA and human cervical squamous cell neoplasms. Cancer Res 41: 3597-3603.

El Shazly MO, Sundberg JP, McPherron T and Smith RD (1985). Enzyme-linked immunosorbent assay for the detection of bovine papillomaviruses. Am J Vet Res 146: 1737-1739.

Eisinger M, Kucarova O, Sarkar NH and Good RA (1975). Propagation of human wart virus in tissue culture. Nature 256: 432-434.

Engel LW, Heilman CA and Howley PM (1983). Transcriptional organisation of bovine papillomavirus type 1. J Virol 47: 516-528.

Favre M, Breitburd F, Croissant O and Orth G (1974). Haemagglutinating activity of bovine papilloma virus. Virology 60: 572-578.

Favre M, Breitburd F, Croissant O and Orth G (1975a). Structural polypeptides of rabbit bovine and human papillomaviruses. J Virol 15: 1239-1247.

Favre M, Orth G, Croissant O and Yaniv M (1975b). Human papillomavirus DNA: Physical map. Proc Natl Acad Sci USA 72: 4810-4814.

Favre M, Breitburd F, Croissant O and Orth G (1977). Chromatin-like structures obtained after alkaline disruption of bovine and human papillomaviruses. J Virol 21: 1205-1209.

Ferenczy A, Mitao M, Nagai N, Silverstein SJ and Crum CP (1985). Latent papillomavirus and recurring genital warts. New Eng J Med 313: 784-788.

Firzlaff JM, Kiviat NB, Beckmann AM, Jenison SA and Galloway DA (1988). Detection of human papillomavirus capsid antigens in various squamous epithelial lesions using antibodies directed against the L1 and L2 open reading frames. Virology 164: 467-477.

Fischer RG and Syverton SJ (1951). The virus-induced papilloma-to-carcinoma sequence. IV. Carcinomas in domestic rabbits infected while in utero. Cancer Res 11: 737-741.

Fleetwood L, Landgren BM and Eneroth P (1984). A method for isolation, characterisation and quantitation of soluble proteins in uterine cervical secretions. Gynecol Obstet Invest 17: 40-46.

Fletcher S and Norval M (1983). On the nature of the deep cellular disturbances in human-papilloma-virus infection of the squamous cervical epithelium. Lancet ii: 546-548.

Follett EAC and Crawford LV (1967). Electron microscope study of the denaturation of human papilloma virus DNA. I. Loss and reversal of supercoiling turns. J Mol Biol 28: 455-459.

Ford JN, Jennings PA, Spradbrow PB and Francis J (1982). Evidence for papillomaviruses in ocular lesions in cattle. Res Vet Sci 32: 257-259.

Forghani B and Schmidt N (1979). Antigen requirements, sensitivity and specificity of enzyme immunoassays for measles and rubella viral antibodies. J Clin Microbiol 9: 657-664.

Forman AB and Prendiville JS (1988). Association of human immunodeficiency virus seropositivity and extensive perineal condylomata accuminata in a child. Arch Dermatol 124: 1010-1011.

Fox EJ, Cook RG, Lewis DE and Rich RR (1986a). Proliferative signals for suppressor T cells. Helper cells stimulated with pokeweed mitogen in vitro produce a suppressor cell growth factor. J Clin Invest 78: 214-220.

Fox PO, Khan PT, McBride BW, McGill SI, and Ward KA (1986b). Tear and serum antibody levels in ocular herpetic infection: diagnostic precision of secretory IgA. Br J Ophthalmol 70: 584-588.

Freed DLJ and Eyres KE (1979). Persistent warts protected from immune attack by a blocking factor. Br J Dermatol 100: 731-733.

Friedl K, Kimura I, Osato T and Ito Y (1970). Studies on a new human cell line (SiHa) derived from carcinoma of uterus. I. Its establishment and morphology. Proc Soc Expl Biol Med 135: 543-545.

Fuchs PG, Girardi F and Pfister H (1988). Human papillomavirus DNA in normal, metaplastic, pre-neoplastic and neoplastic epithelia of the cervix uteri. Int J Cancer 41: 41-45.

Fuchs PG, Girardi F and Pfister H (1989). Human papillomavirus 16 DNA in cervical cancers and in lymph nodes of cervical cancer patients : a diagnostic marker for early metastases? Int J Cancer 43: 41-44.

Fuchs PG, Iftner T, Weninger J and Pfister H (1986). Epidermodysplasia verruciformis associated human papillomavirus 8: genomic sequence and comparative analysis. J Virol 58: 626-634.

Gabel H, Jontell M, Ohman C and Brynger H (1987). Epidermal Langerhans cells in the early phase of immunosuppression. Transplant Proc 19: 1205-1206.

Gallagher CH, Canfield PJ, Greenoak GE and Reeve VE (1984). Characterisation and histogenesis of tumours in the hairless mouse produced by low-dosage incremental ultraviolet radiation. J Invest Dermatol 83: 169-174.

Galloway DA and McDougall JK (1983). The oncogenic potential of herpes simplex viruses: evidence for a "hit and run" mechanism. Nature 302: 21-24.

Gassenmaier A, Pfister H and Hornstein OP (1986). Human papillomavirus 25-related DNA in solitary keratoacanthoma. Arch Dermatol Res 79: 73-76.

Gatter KC, Pulford KAF, Vanstapel MJ, Roach B, Mortimer P, Woolston RE, Taylor Papadimitriou J, Lane EB and Mason DY (1984a). an immunohistological study of benign and malignant skin tumours: epithelial aspects. Histopathol 8: 209-227.

Gatter KC, Morris HB, Roach B, Mortimer P, Fleming KA and Mason DY (1984b). Langerhans cells and T cells in human skin tumours: an immunohistological study. Histopathol 8: 229-244.

Gawkrodger DJ, Carr MM, McVittie E, Guy K and Hunter JAA (1987). Keratinocyte expression of MHC Class II antigens in allergic sensitisation and challenge reactions and in irritant contact dermatitis. J Invest Dermatol 88: 11-16.

Genner J (1971). Localisation of wart virus antigen in human plantar warts (Verrucae vulgaris). Acta Dermatovenereol (Stockholm) 51: 361-364.

Gifford GE and Flick DA (1987). Natural production and release of tumour necrosis factor. In "Tumour necrosis factor and related cytotoxins": CIBA Foundation Symposium 131: 3-20.

Giri I, Danos O and Yaniv M (1985). Genomic structure of the cottontail rabbit (Shope) papillomavirus. Proc Natl Acad Sci USA 82: 1580-1584.

Giri I and Danos O (1986). Papillomavirus genomes: from sequence data to biological properties. Trends in Genetics 2: 227-232.

Giri I and Yaniv M (1988). Study of the E2 gene prodict of the cottontail rabbit papillomavirus reveals a common mechanism of transactivation among papillomaviruses. J Virol 62: 1573-1581.

Gissmann L (1984). Papillomaviruses and their association with cancer in animals and in man. Cancer Surv 3: 161-181.

Gissmann L, De Villiers EM and zur Hausen H (1982a). Analysis of human genital warts (Condylomata accuminata) and other genital tumours for human papillomavirus type 6 DNA. Int J Cancer 29: 143-146.

Gissmann L, Diehl V, Schultz-Coulon HJ and zur Hausen H (1982b). Molecular cloning and characterisation of human papillomavirus DNA derived from a laryngeal papilloma. J Virol 44: 393-400.

Gissmann L, Pfister H and zur Hausen H (1977). Human papilloma viruses (HPV): characterisation of four different isolates. Virology 76: 569-580.

Gissmann L and Schwarz E (1986). Persistence and expression of human papillomavirus DNA in genital cancer. In "Papillomaviruses": CIBA Foundation Symposium 120: 190-197.

Gissmann L, Wolnik L, Ikenberg H, Koldovsky U, Schnurch H-G and zur Hausen H (1983). Human papillomavirus types 6 and 11 sequences in genital and laryngeal papillomas and in some cervical cancers. Proc Nat Acadl Sci USA 80: 560-563.

Gissmann L and zur Hausen H (1976). Human papillomavirus DNA: physical mapping and genetic heterogeneity. Proc Natl Acad Sci USA 73: 1310-1313.

Glinski W, Obalek S, Jablonska S and Orth G (1981). T cell defect in patients with epidermodysplasia verruciformis due to human papillomavirus type 3 and 5. Dermatologica 162: 141-147.

Gloss B, Bernard HU, Seedorf K and Klock G (1987). The upstream regulatory region of the human papillomavirus-16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones. EMBO J 6: 3735-3743.

Grail A and Norval M (1988). Significance of smoking and detection of serum antibodies to cytomegalovirus in cervical dysplasia. Br J Obstet Gynecol 95: 1103-1110.

Greenspan D, Greenspan JS, Conant M, Petersen V, Silverman S and de Souza Y (1984). Oral "hairy" leukoplakia in male homosexuals: evidence of association with both papillomavirus and a herpes-group virus. Lancet ii: 831-834.

Groff DE, Mitra R and Lancaster W (1986) cited by Pfister H, Krubke J, Dietrich W, Iftner T and Fuchs PG In Classification of the papillomaviruses - mapping the genome. CIBA Foundation Symposium 120: 3-14 and by Baker CC (1987). "The genomes of the papillomaviruses". 6th International Papilloma Workshop: Short review of current literature, 11-36.

Gross G, Hagedorn M, Ikenberg H, Rufli T, Dahlet C, Grosshans E and Gissmann L (1985a). Bowenoid papulosis: presence of human papillomavirus (HPV) structural antigens and of HPV-16 related DNA sequences. Arch Dermatol 121: 858-863.

Gross G, Ikenberg H, Gissmann L and Hagedorn M (1985b). Papillomavirus infection of the anogenital region: correlation between histology, clinical picture and virus type. Proposal of a new nomenclature. J invest Dermatol 85: 147-152.

Gross G, Schneider A and Gissmann L (1985c). Unpublished data, cited Gissmann L and Gross G. Clin Dermatol 3: 126.

Grubb GS (1986). Human papillomavirus and cervical neoplasia: epidemiological considerations. Int J Epidemiol 15: 1-7.

Grussendorf-Conen E-I (1985). Condylomata accuminata. Clin Dermatol 3: 97-103.

Guillet G, Borredon J and Duboseq M-F (1987). Prevalence of warts on hands of poultry slaughterers and poultry warts. Arch Dermatol 123: 718-719.

Haase A, Brahic M, Stowring L and Blum H (1984). Detection of viral nucleic acids by in situ hybridisation. Methods Virol 7: 189-226.

Haftek M, Jablonska S, Szymanczyk J and Jarzabek-Chorzelska M (1987). Langerhans cells in epidermodysplasia verruciformis. Dermatologica 174: 173-179.

Hampar B and Ellison SA (1961). Chromosomal aberrations induced by an animal virus. Nature 192: 145-147.

Hanna N (1985). The role of natural killer cells in the control of tumour growth and metastasis. Biochim Biophys Acta 780: 213-226.

Harris RWC, Brinton LA, Cowdell RH, Skegg DCG, Smith PG, Vessey MP and Doll R (1980). Characteristics of women with dysplasia or carcinoma in situ of the cervix uteri. Br J Cancer 42: 359-367.

Hauser H, Gross G, Schneider A, de Villiers E-M, Gissmann L and Wagner D (1985). HPV-16 related bowenoid papulosis. Lancet ii: 106.

Hawthorn RJS, Murdoch JB, Maclean AB and Mackie RM (1988). Langerhans' cells and subtypes of human papillomavirus in cervical intraepithelial neoplasia. Br Med J 297: 643-646.

Heilman CA, Law MF, Israel MA and Howley PM (1980). Cloning of human papilloma virus genomic DNAs and analysis of homologous poly-nucleotide sequences. J Virol 36: 395-407.

Hellstrom I, Evans CA and Hellstrom KE (1969). Cellular immunity and its serum-mediated inhibition in Shope-virus-induced rabbit papillomavirus. Int J Cancer 4: 601-607.

Herberman JR and Ortaldo RB (1981). Natural killer cells : their role in defenses against disease. Science 214: 24-30.

Hilliard GD, Massey FM and O'Toole RV (1979). Vulvar neoplasia in the young. Am J Obstet Gynecol 135: 185-188.

Hills E and Laverty CR (1979). Electron microscopic detection of papillomavirus particles in selected koilocytotic cells in a routine cervical smear. Acta Cytol 23: 53-56.

Holgate CS, Jackson P, Pollard K, Lunny D and Bird CC (1986). Effect fixation on T and B lymphocyte surface membrane antigen demonstration in paraffin processed tissue. J Pathol 149: 293-300.

Howie SEM, Norval M and Maingay JP (1986). Alterations in epidermal handling of HSV-1 antigens *in vitro* induced by *in vivo* exposure to UV-B light. Immunology 57: 225-230.

Hsu S, Raine L and Fanger H (1981). A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am J Clin Pathol 75: 734-738.

Huang WM, Gibson P, Qu J and Polsk JM (1983). Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-lysine as a slide coating. Histochemistry 77: 275-279.

Hughes R.G. (1988). "The local immune response to human virus infection and neoplastic disease of the uterine cervix". M.D. Thesis University of Edinburgh.

Hughes RG, Neill WA and Norval M (1987). Nuclear DNA analysis of koilocytic and premalignant lesions of the uterine cervix. Br Med J 294: 267-269.

Hughes RG, Norval M and Howie SEM (1988). The expression of major histocompatibility class II antigens by Langerhans cells in cervical intraepithelial neoplasia. J Clin Pathol 41: 253-259.

Iftner T, Bierfelder S, Csapo Z and Pfister H (1988). Involvement of human papillomavirus type 8 genes E6 and E7 in transformation and replication. J Virol 62: 3655-3661.

Ikenberg H, Gissmann L, Gross G, Grussendorf-Conen E-I and zur Hausen (1983). Human papillomavirus type-16-related DNA in genital Bowen's disease and in Bowenoid papulosis. Int J Cancer 32: 563-565.

Ionesco M, Baudenon S, Barrasso R, Buu-Hoi P, Behret T, Moniot-Vittoz M, Sadoul G, de Brux S, Coupez F and Orth G (1987). Histological aspects of lesions of the cervix uteri correlated with different types of human papillomavirus. Bull Cancer (Paris) 74: 397-406.

Ito Y and Evans CA (1961). Induction of tumours in domestic rabbits with nucleic acid preparations from partially purified Shope papillomavirus and from extracts of the papillomas of domestic and cottontail rabbits. J Exp Med 114: 485-500.

Ivanyi L and Morison WL (1976). *In vitro* lymphocyte stimulation by wart antigen in man. Br J Dermatol 94: 523-527.

Iwasaka T, Sheridan JF and Aurelian L (1983). Immunity to herpes simplex virus type 2: recurrent lesions are associated with the induction of suppressor cells and soluble suppressor factors. Infect Immun 42: 955-964.

Iwatsuki K, Tagami H, Takigawa M and Yamada M (1986). Plane warts under spontaneous regression. Arch. Dermatol. 122: 655-659.

Jablonska S (1986). Epidermodysplasia verruciformis: a model for understanding the oncogenicity of human papillomavirus. In "Papillomaviruses": CIBA Foundation Symposium 120: 171.

Jablonska S, Dabrowski J and Jakubowicz K (1972). Epidermodysplasia verruciformis as a model in studies on the role of papovaviruses in oncogenesis. Cancer Res 32: 583-589.

Jablonska S, Kawashima M, Obalek S, Szyumanczyk J and Orth G (1987). Human papillomavirus-related cutaneous benign lesions and skin malignancies. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 309-317.

Jablonska S, Obalek S, Orth G, Haftek M, Jarzabek-Chorzelska M (1982a). Regression of the lesions of epidermodysplasia verruciformis. Br J Dermatol 107: 109-116.

Jablonska S and Orth G (1985a). Epidermodysplasia verruciformis. Clin Dermatol 3: 83-96.

Jablonska S, Orth G, Glinski G, Obalek S, Jarzabek-Chorzelska M, Croissant O, Favre M and Rzesa G (1980). Leukaemia, lymphomas and papillomas: comparative aspects. Ed. P.A. Backman, 107-131.

Jablonska S, Orth G and Lutzner MA (1982b) Immunopathology of papillomavirus-induced tumours in different tissues. Springer Semin Immunopathol 5: 33-62.

Jablonska S, Orth G, Obalek S, Croissant O, Jarzabek-Chorzelska M and Kremsdorf D (1983). Oncogenic potential of human papillomaviruses epidermodysplasia verruciformis: A counterpart of Shope papilloma: carcinoma complex. Arch Geschwulstforsch 53: S207-S215.

Jablonska S, Orth G, Obalek S and Croissant O (1985). Cutaneous warts: clinical histologic and virologic correlations. Clin Dermatol 3: 71-82.

Jablonski E, Moomaw EW, Tullis RH and Ruth JL (1986). Preparation of oligonucleotide-alkaline phosphatase conjugates and their use as hybridisation probes. Nucleic Acid Res 14: 6115-6128.

Jakobsen A, Kristensen PB and Poulsen HK (1983). Flow cytometric classification of biopsy specimens from cervical intra-epithelial neoplasia. Cytometry 4: 166-169.

Jarrett WFH, McNeill PE, Grimshaw WTR, Selman IE and McIntyre WIM (1978). High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papillomavirus. Nature 274: 215-217.

Jarrett WFH, Campo MS, O'Neil BW, Laird HM and Coggins LW (1984). A new bovine papillomavirus (BPV-6) causing true epithelial papillomas of the mammary gland skin: a member of a proposed new BPV subgroup. Virology 136: 255-264.

Jarrett WFH (1985). Bovine Papillomaviruses. Clin Dermatol 3: 8-19.

Jarrett WFH (1987). Environmental carcinogens and papillomaviruses in the pathogenesis of cancer. Proc Roy Soc B 231: 1-11.

Jayant K (1987). Additive effect of two risk factors in the aetiology of cancer of the cervix uteri. Br J Cancer 56: 685-686.

Jenison SA, Firzlaff JM, Langenberg A and Galloway DA (1988). Identification of immunoreactive antigens of human papillomavirus type 6b by using Escherichia coli-expressed fusion proteins. J Virol 62: 2115-2123.

Jenson AB, Rosenthal JD, Olson C, Pass F, Lancaster WD and Shah K (1980). Immunologic relatedness of papillomaviruses from different species. J Natl Cancer Inst 64: 495-498.

Julius MH, Simpson E and Herzenberg LA (1973). A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur J Immunol 3: 645-649.

Kahn T, Schwarz E and zur Hausen H (1986). Molecular cloning and characterisation of the DNA of a new human papillomavirus (HPV-30) from a laryngeal carcinoma. Int J Cancer 37: 61-65.

Kalimo K, Terho P, Honkonen E, Gronroos M and Halonen O (1981). Chlamydia trachomatis and herpes simplex virus IgA antibodies in cervical secretions of patients with cervical atypia. Br J Obstet Gynecol 88: 1130-1134.

Kaminski M, Pawinska M, Jablonska S, Szmurko A, Majewski S and Orth G (1985). Increased natural killer cell activity in patients with epidermodysplasia verruciformis. Arch Dermatol 121: 84-86.

Kanda T, Furuno A and Yoshiike K (1988). Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. J Virol 62: 610-613.

Kasher MS and Roman A (1988). Alteratio ns in the regulatory region of the human papillomavirus type 6 genome are generated during propagation in Escherichia coli. J Virol 62: 3295-3300.

Katz SI, Cooper KD, Iijima M and Tsuchida T (1985). The role of Langerhans cells in antigen presentation. J Invest Dermatol 85: 96S-98S.

Katz RL, Veanattukalathil S and Weiss KM (1987). Human papillomavirus infection and neoplasia of the cervix and anogenital region in women with Hodgkin's disease. Acta Cytol 31: 845-854.

Kaur P and McDougall JK (1988). Characterisation of primary human keratinocytes transformed by human papillomavirus type 18. J Virol 62: 1917-1924.

Kent C, Samuel M and Winkelstein W (1987). The role of anal/genital warts in HIV infection. J Am Med Assoc 258: 3385-3386.

Kienzler JL, Lemoine MT, Orth G, Jibard N, Blanc D, Laurent R and Agache P (1983). Humoral and cell-mediated immunity to human papillomavirus type 1 (HPV-1) in human warts. Br J Dermatol 108: 665-672.

Kinlen LJ, Sheil AGR, Peto J and Doll R (1979). Collaborative United Kingdom and Australasian study of cancer in patients treated with immunosuppressive drugs. Br Med J ii: 1461-1466.

Kirchner H (1986). Immunobiology of human papillomavirus infection. Prog Med Virol 33: 1-41.

Kleinschmidt AK, Kass SJ, Williams RC and Knight CA (1965). Cyclic DNA of Shope papillomavirus. J Mol Biol 13: 749-756.

Klug A and Finch JT (1965). Structure of viruses of the papilloma-polyoma type 1 human wart virus. J Mol Biol 11: 403-423.

Komly CA, Breitburd F, Croissant O and Streeck RE (1986). The L2 open reading frame of human papillomavirus type 1a encodes a minor structural protein carrying type-specific antigens. J Virol 60: 813-816.

Koranda FC, Dehmel EI, Khan G and Penn I (1974). Cutaneous complications in immunosuppressed renal homograft recipients. J Am Med Assoc 229: 419-424.

Koss LG and Durfee GR (1956). Unusual patterns of squamous epithelium of the uterine cervix and pathologic study of koilocytic atypia. Ann NY Acad Sci 63: 1245-1261.

Kozma R and Adinolfi M (1987). In situ hybridisation and the detection of biotinylated DNA probes. Mol Biol Med 4: 357-364.

Kreider JW (1963). Studies on the mechanism responsible for the spontaneous regression of the Shope rabbit papilloma. Cancer Res 23: 1593-1599.

Kreider JW and Bartlett GL (1981). The Shope papilloma-carcinoma complex in rabbits: a model system of neoplastic progression and spontaneous regression. Adv. Cancer Res 35: 81-110.

Kreider JW and Bartlett GL (1985). Shope rabbit papilloma-carcinoma complex. A model system of HPV infections. Clin Dermatol 3: 20-26.

Kreider JW, Bartlett GL and Sharkey FE (1979). Primary neoplastic transformation *in vivo* of xenogeneic skin grafts on nude mice. Cancer Res 39: 273-276.

Kreider JW, Haft HM and Roode PB (1971). Growth of human skin on the hamster. J Invest Dermatol 57: 66-71.

Kreider JW, Howett MK, Wolfe SA, Bartlett GL, Zaino RJ, Sedlacek TV and Mortel R (1985). Morphological transformation *in vivo* of human uterine cervix with papillomavirus from condylomata acuminata. Nature 317: 639-641.

Kreider JW, Howett MK, Lill NL, Bartlett GL, Zaino RJ, Sedlacek TV and Mortel R. (1986). *In vivo* transformation of human skin with human papillomavirus type 11 from condylomata acuminata. J Virol 59: 369-376.

Kreider JW, Howett MK, Leure-Dupre AE, Zaino RJ and Weber JA (1987). Laboratory production *in vivo* of infectious human papillomavirus type 11. J Virol 61: 590-593.

Kremsdorf D, Jablonska S, Favre M and Orth G (1983). Human papillomaviruses associated with epidermodysplasia verruciformis. II Molecular cloning and biochemical characterisation of human papillomavirus 3a, 8, 10 and 12 genomes. J Virol 48: 340-351.

Kremsdorf D, Favre M, Jablonska S, Obalek S, Rueda AL, Lutzner MA, Blanchet-Bardon C, Van Voorst Vader PC and Orth G (1984). Molecular cloning and characterisation of the genomes of nine newly recognised human papillomavirus types associated with epidermodysplasia verruciformis. J Virol 52: 1012-1018.

Kurnick JT, Ostberg L, Stegagno M, Kimura AK, Orn A and Sjoberg O (1979). A rapid method for the separation of functional lymphoid cell populations of human and animal origin on PVP-silicon (Percoll) density gradients. Scand J Immunol 10: 563-573.

Lack EE, Jenson AB, Smith HG, Healy GB, Pass F and Vawter GF (1980). Immunoperoxidase localisation of human papillomavirus in laryngeal papillomas. Intervirology 14: 148-154.

Lampert IA (1984). Expression of HLA-DR (Ia-like) antigen on epidermal keratinocytes in human dermatoses. Clin exp Immunol 57: 93-100.

Lancaster WD (1979). Physical maps of bovine papillomavirus type 1 and type 2 genomes. J Virol 32: 684-687.

Lancaster WD, Castellano C, Santos C, Delgado G, Kurman RJ and Jenson AB (1986). Human papillomavirus deoxyribonucleic acid in cervical carcinoma from primary and metastatic sites. Am J Obstet Gynecol 154: 115-119.

Lancaster WD and Meinke W (1975). Persistence of viral DNA in human cell cultures infected with human papillomavirus. Science 256: 434-436.

Lancaster WD, Olson C and Meinke W (1976). Quantitation of bovine papilloma viral DNA in viral-induced tumours. J Virol 17: 824-831.

Lancaster WD, Olson C and Meinke W (1977). Bovine papilloma virus : presence of virus-specific DNA sequences in naturally occurring equine tumours. Proc Natl Acad Sci (USA) 74: 524-528.

Lancaster WD and Olson C (1978). Demonstration of two distinct classes of bovine papilloma virus. Virology 89: 372-379.

Lancaster WD and Olson C (1982). Animal papillomaviruses. Microbiol Rev 46: 191-207.

Langer PR, Waldorp AA and Ward DC (1981). Enzymatic synthesis of biotin-labelled polynucleotides: Novel nucleic acid affinity probes. Proc Natl Acad Sci USA 78: 6633-6637.

Lanzavecchia A, Ferrarini M and Celada F (1982). Human T cell lines with antigen specificity and helper activity. Eur J Immunol 12: 468-474.

La Porta RF and Taichman LB (1982). Human papillomaviral DNA replicates as a stable episome in cultured epidermal keratinocytes. Proc Natl Acad Sci USA 79: 3393-3397.

Laurent R, Kienzler JL, Croissant O and Orth G (1982). Two anatomo-clinical types of warts with plantar localisation: specific cytopathogenic effects of papillomavirus. Arch Dermatol Res 274: 101-111.

La Vecchia C, Franceschi S, Decarli A, Fasoli M, Gentile A and Tognoni G (1986). Cigarette smoking and the risk of cervical neoplasia. Am J Epidemiol 123: 22-29.

Laverty C (1979). Non-condylomatous wart virus infection of the cervix: cytologic, histologic and electronmicroscopic features. Obstet Gynecol Surv 34: 720-722.

Law MF, Lancaster WD and Howley PM (1979). Conserved polynucleotide sequences among the genomes of papillomaviruses. J Virol 32: 199-207.

Law MF, Lowy DR, Dvoretsky I and Howley PM (1981). Mouse cells transformed by bovine papillomavirus contain only extra-chromosomal viral DNA sequences. Proc Natl Acad Sci USA 78: 2727-2731.

Leary JJ, Brigati DJ and Ward DC (1983). Rapid and sensitive colormetric method for visualising biotin-labelled DNA probes hybridised to DNA or RNA immobilised on nitrocellulose: Dot blots. Proc Natl Acad Sci USA 80: 4045-4049.

Le Bouvier GL, Sussman M and Crawford LV (1966). Antigenic diversity of mammalian papillomaviruses. J Gen Microbiol 45: 497-501.

Lee AKY and Eisinger RM (1976). Cell-mediated immunity (CMI) to human wart virus and wart-associated tissue antigens. Clin Exp Immunol 26: 419-424.

Lee KP and Olson C (1968). Response of calves to intravenous and repeated intradermal inoculation of bovine papilloma virus. Am J Vet Res 29: 2103-2112.

Lee KP and Olson C (1969). A gel diffusion precipitin test for bovine papilloma virus. Am J Vet Res 30: 725-731.

Lehn H, Krieg P and Sauer G (1985). Papillomavirus genomes in human cervical tumours: analysis of their transcriptional activity. Proc Natl Acad Sci USA 82: 5540-5544.

Lehner T, Lamb JR and Kontiainen S (1982). Cell-bound helper and suppressor factors in primate lymphocytes. Clin Exp Immunol 47: 706-714.

Lewis FA, Griffiths S, Dunnicliff R, Wells M, Dudding N and Bird CC (1987). Sensitive in situ hybridisation technique employing biotin-streptavidin-polyalkaline phosphatase complex. J Clin Pathol 40: 163-166.

Li C-CH, Gilden RV, Showalter ST and Shah KV (1988). Identification of the human papillomavirus E2 protein in genital tract tissues. J Virol 62: 606-609.

Li C-CH, Shah KV, Seth A and Gilden RV (1987a). Identification of human papillomavirus type 6b L1 open reading frame protein in condylomas and corresponding antibodies in human sera. J Virol 61: 2684-2690.

Li P, Medon PP, Skingle DC, Langer JA and Symons RH (1987b). Enzyme-linked synthetic oligonucleotide probes: non-radioactive detection of enterotoxigenic Escherichia coli in faecal specimens. Nucleic Acid Res 15: 5275-5287.

Lin HJ, Wi P-C and Lai C-L (1987). An oligonucleotide probe for the detection of hepatitis B virus DNA in serum. J Virol Methods 15: 139-149.

Lodi A, Betti R, Cataneo M, Rosti A, Masnada MC, Marmini A and Crosti C (1987). A comparative study on peripheral blood lymphocyte subpopulations in different kinds of warts. Acta Derm Venereol 67: 169-172.

Loning T, Ikenberg H, Becker J, Gissmann L, Hoepfer J and zur Hausen H (1985). Analysis of oral papillomas, leukoplakias, and invasive carcinomas for human papillomavirus type related DNA. J Invest Dermatol 84: 417-420.

Lorincz AT, Lancaster WD and Temple GF (1986). Cloning and characterisation of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J Virol 58: 225-229.

Lorincz AT, Temple GF, Kurman RJ, Jenson AB and Lancaster WD (1987). Oncogenic association of specific human papillomavirus types with cervical neoplasia. J Natl Cancer Inst 79: 671-677.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275.

Lowy DR, Dvoretsky I, Shober R, Law M-F, Engel L and Howley PM (1980). In vitro tumorigenic transformation by a defined sub-genomic fragment of bovine papilloma virus DNA. Nature 287: 72-74.

Lusky M and Botchan MR (1985). Genetic analysis of bovine papillomavirus type 1 trans-acting replication factors. J Virol 53: 955-965.

Lutzner M, Croissant O, Ducasse M-F, Kreis H, Crosnier J and Orth G (1980). A potentially oncogenic human papillomavirus (HPV-5) found in two renal allograft recipients. J Invest Dermatol 75: 353-356.

Lutzner M, Kuffer R, Blanchet-Bardon C and Croissant O (1982). Different papillomaviruses as the causes of oral warts. Arch Dermatol 118: 393-399.

Lutzner MA, Orth G, Dutronquay V, Ducasse M-F, Kreis H and Crosnier J (1983). Detection of human papillomavirus type 5 DNA in skin cancers of an immunosuppressed renal allograft recipient. Lancet ii: 422-424.

Lutzner MA, Blanchet-Bardon C and Orth G (1984). Clinical observations, virologic studies, and treatment trials in patients with epidermodysplasia verruciformis, a disease induced by specific human papillomaviruses. J Invest Dermatol 83: 18s-25s.

McArdle JP and Muller HK (1986). Quantitative assessment of Langerhans cells in human cervical intraepithelial neoplasia and wart virus infection. Am J Obstet Gynecol 154: 509-515.

McCance DJ (1986). Human papillomaviruses and cancer. Biochim Biophys Acta 823: 195-205.

McCance DJ (1988). News on papillomaviruses. Nature 335: 765-766.

McCance DJ, Campion MJ, Clarkson PK, Chesters PM, Jenkins D and Singer A (1985a). Prevalence of human papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and invasive carcinoma of the cervix. Br J Obstet Gynaecol 92: 1101-1105.

McCance DJ, Clarkson PK, Dyson JL, Walker PG and Singer A (1985b). Human papillomavirus types 6 and 16 in multifocal intra-epithelial neoplasia of female lower genital tract. Br J Obstet Gynaecol 92: 1093-1100.

McCance DJ, Walker PG, Dyson JL, Coleman DV and Singer A (1983). Presence of human papillomavirus DNA sequences in cervical intraepithelial neoplasia. Br Med J 287: 784-788.

McDougall JK, Galloway DA and Fenoglio CM (1980). Cervical carcinoma: detection of herpes simplex virus RNA in cells undergoing neoplastic change. Int J Cancer 25: 1-8.

McMichael H (1967). Inhibition by methyl prednisolone of regression of the Shope rabbit papilloma. J Natl Cancer Inst 39: 55-65.

Macnab JCM, Walkinshaw JA, Cordiner JW and Clements JB (1986). Human papillomavirus in clinically and histologically normal tissue of patients with genital cancer. New Eng J Med 315: 1052-1058.

Maddox PH and Jenkins D (1987). 3-Aminopropyltriethoxysilane (APES): a new advance in section adhesion. J Clin Pathol 40: 1256-1260.

Maitland NJ, Kinross JH, Busuttil A, Ludgate SM, Smart GE and Jones KW (1981). The detection of DNA tumour virus-specific RNA sequences in abnormal human cervical biopsies by in situ hybridisation. J Gen Virol 55: 123-137.

Maitland NJ, Cox MF, Lynas C, Prime SS, Meanwell CA and Scully C (1987). Detection of human papillomavirus DNA in biopsies of human oral tissue. Br J Cancer 56: 245-250.

Malejczyk J, Majewski S, Jablonska S and Orth G (1987). Natural cell-mediated cytotoxicity in patients with anogenital lesions induced by potentially oncogenic human papillomaviruses. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 381-385.

Malejczyk J, Majewski S, Jablonska S, Rogozinski T and Orth G (1989). Abrogated NK-cell lysis of human papillomavirus (HPV)-16-bearing keratinocytes in patients with pre-cancerous HPV-induced anogenital lesions. Int J Cancer 43: 209-214.

Malgouyat J (1983). New approach to the treatment of genital condyloma in women. Gynecologie 34: 415-417.

Maniatis T, Fritsch EF and Sambrook J (1982). Molecular cloning: a laboratory model. Cold Spring Harbour (NY) Laboratory.

Marshall V (1974). Premalignant and malignant skin tumours in immunosuppressed patients. Transplantation 17: 272-275.

Martinez O (1969). Relationship of squamous cell carcinoma of the cervix uteri to squamous cell carcinoma of the penis. Cancer Res 24: 777-780.

Martz E and Howell DM (1989). CTL : virus control cells first and cytolytic cells second. Immunol Today 10: 79-86.

Mason DY, Krissansen GW, Davey FR, Crumpton MJ and Gatter KC (1988). Antisera against epitopes resistant to denaturation on T3 (CD3) antigen can detect reactive and neoplastic T cells in paraffin embedded tissue biopsy specimens. J Clin Pathol 41: 121-127.

Massing AM and Epstein WL (1963). Natural history of warts. A two year study. Arch Dermatol 87: 306-310.

Matlashewski G, Banks L, Wu-Liao J, Spence P, Pim D and Crawford L (1986). The expression of human papillomavirus type 18 E6 protein in bacteria and the production of anti-E6 antibodies. J Gen Virol 67: 1909-1916.

Matlashewski G, Osborn K, Banks L, Stanley M and Crawford L (1988). Transformation of primary human fibroblast cells with human papillomavirus type 16 DNA and EJ-ras. Int J Cancer 42: 232-238.

Matlashewski G, Schneider J, Banks L, Jones N, Murray A and Crawford L (1987). Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. EMBO J 6: 1741-1746.

Matthews RS and Shirodaria PV (1973). Study of regressing warts by immunofluorescence. Lancet i: 689-691.

Meanwell CA, Cox MF, Blackledge G and Maitland NJ (1987). HPV-16 DNA in normal and malignant cervical epithelium: implications for the aetiology and behaviour of cervical neoplasia. Lancet i: 703-707.

Mehra V, Mason LH, Rothman W, Reinherz E, Schlosiman SF and Bloom BR (1980). Delineation of a human T cell subset responsible for lepromin-induced suppression in leprosy patients. J Immunol 125: 1183-1187.

Meinke W and Meinke GC (1981). Isolation and characterisation of the major capsid protein of bovine papillomavirus type 1. J Gen Virol 52: 15-24.

Meinkoth J and Wahl G (1984). Hybridisation of nucleic acids immobilised on solid supports. Anal Biochem 138: 267-284.

Meisels A and Fortin R (1976). Condylomatous lesions of the cervix and vagina. I. Cytologic patterns. Acta Cytol 20: 505-509.

Meisels A, Fortin R and Roy M (1977). Condylomatous lesions of the cervix. II. Cytologic, colposcopic and histopathologic study. Acta Cytol 21: 379-390.

Meisels A, Roy M, Fortier M, Morin C, Casas-Cordero M, Shah KV and Turgeon H (1981). Human papillomavirus infection of the cervix. Acta Cytol 25: 7-16.

Merriman H, Woods S, Winter C, Fahnlander A and Corey L (1984). Secretory IgA antibody in cervicovaginal secretions from women with genital infection due to herpes simplex virus. J Infect Dis 149: 505-510.

Michnovicz JJ, Herschcopf RJ, Naganuma W, Bradlow HL and Fishman J (1986). Increased 2-hydroxylation of estradiol as a possible mechanism for the anti-estrogenic effect of cigarette smoking. New Eng J Med 315: 1305-1309.

Mims CA (1987). "The Pathogenesis of Infectious Disease" 3rd Edition. Academic Press.

Moar MH, Campo MS, Laird H and Jarrett WFH (1981). Peristence of non-integrated viral DNA in bovine cells transformed *in vitro* by bovine papillomavirus type 2. Nature 293: 749-751.

Moar MH, Jarrett WFH and O'Neil BW (1986). Viral DNA sequences detected in a hamster liposarcoma induced by bovine papilloma-virus type 4. J Gen Virol 67: 187-190.

Mohanty KC and Scott CS (1986). Immunotherapy of gential warts with inosine pranabex (Imunovir): preliminary study. Genitourinary Med 62: 352-355.

Morhenn V (1988). Keratinocyte proliferation in wound healing and skin diseases. Immunol Today 9: 104-106.

Morhenn V, Nickoloff BJ and Mansbridge JN (1985). Induction of the synthesis of triton-soluble proteins in human keratinocytes by gamma interferon. J Invest Dermatol 85: 27s - 29s.

Morin C, Braun L, Casas Cordero M, Shah KV, Roy M, Fortier M and Meisels A (1981). Confirmation of the papillomavirus etiology of condylomatous cervix lesions by the peroxidase-anti-peroxidase technique. J Natl Cancer Inst 66: 831-835.

Morin C and Meisels A (1980). Papilloma virus infection of the uterine cervix. Acta Cytol 24: 82-84.

Morison WL (1975a). In vitro assay of immunity to human wart antigen. Br J Dermatol 93: 545-552.

Morison WL (1975b). Viral warts, herpes simplex and herpes zoster in patients with secondary immune deficiencies and neoplasms. Br J Dermatol 92: 625-630.

Morison WL (1975c). Cell-mediated immune responses in patients with warts. Br J Dermatol 93: 553-556.

Morris HHB, Gatter KC, Sykes C, Casemore V and Mason DY (1983). Langerhans cells in human cervical epithelium: effects of wart virus infection and intraepithelial neoplasia. Br J Obstet Gynaecol 90: 412-420.

Mose Larsen P , Storgaard L and Fey SJ (1987). Proteins present in bovine papillomavirus particles. J Virol 61: 3596-3601.

Mould RF and Barrett A (1976). New primary cancers in patients originally presenting with carcinoma of the cervix. Br J Obstet Gynecol 83: 81-85.

Mounts P, Shah KV and Kashima H (1982). Viral aetiology of juvenile- and adult-onset squamous papilloma of the larynx. Proc Natl Acad Sci USA 79: 5425-5429.

Muller H and Gissmann L (1978). Mastomys natalensis papilloma virus (MnPV), the causative agent of epithelial proliferation: characterisation of the virus particle. J Gen Virol 41: 315-323.

Munoz N, Bosch X and Kaldor JM (1988). Does human papillomavirus cause cervical cancer? The state of the epidemiological evidence. Br J Cancer 57: 1-5.

Murdoch JB, Cassidy LJ, Fletcher K, Cordiner JW and Macnab JCM (1988). Histological and cytological evidence of viral infection and human papilloma virus type 16 DNA sequences in cervical intraepithelial neoplasia and normal tissue in the west of Scotland: evaluation of treatment policy. Br Med J 296: 381-385.

Murphy GF and Harrist TJ (1982). Langerhans' cells and identification with antibodies. Arch Pathol Lab Med 106: 255.

Mustafa AS and Godal T (1985). BCG-induced suppressor T cells optimal conditions for in vitro induction and mode of action. Clin Exp Immunol 62: 474-481.

Naghashfar Z, Sawada E, Kutcher MJ, Swancar J, Gupta J, Daniel R, Kashima H, Woodruff JD and Shah K (1985). Identification of genital tract papillomaviruses HPV-6 and HPV-16 in warts of the oral cavity. J Med Virol 17: 313-324.

Nakai Y, Lancaster WD, Lim LY and Bennett Jenson A (1986). Monoclonal antibodies to genus and type specific papilloma virus structural antigens. Intervirology 25: 30-37.

Nasseri M and Wettstein FO (1984a). Differences exist between viral transcripts in cottontail rabbit papillomavirus-induced benign and malignant tumours as well as non-virus producing and virus producing tumours. J Virol 51: 706-712.

Nasseri M and Wettstein FO (1984b). Cottontail rabbit papillomavirus specific transcripts in transplantable tumours with integrated DNA. Virology 138: 362-367.

Navarette C, Jaraquemada D, Fainboim L, Karr R, Hui K, Awad J, Bagnara M and Festenstein H (1985). Genetic and functional relationship of the HLA-DR and HLA-DQ antigens. Immunogenetics 21: 97-107.

Neary K, Horwitz BH and Di Maio D (1987). Mutational analysis of open reading frame E4 of bovine papillomavirus type 1. J Virol 61: 1248-1252.

Neill WA (1984). Cell mediated immune responses to cytomegalovirus in patients with abnormalities of the uterine cervix. M.Phil Thesis, University of Edinburgh.

Neill WA and Norval M (1984). Natural killer cell activity in patients with abnormalities of the uterine cervix. Gynecol Obstet Invest 18: 122-128.

Neill WA and Norval (1985). Cell-mediated immune responses to cytomegalovirus in patients with dysplasia of the uterine cervix. Gynecol Obstet Invest 20: 96-102.

Nickoloff BJ, Basham TY, Merigan TC, Torseth JW and Morhenn VB (1986). Human keratinocyte-lymphocyte reactions in vitro. J Invest Dermatol 87: 11-18.

Niimura M, Pass F, Wooley R and Soutor CA (1975). Primary tissue culture of human wart-derived epidermal cells (keratinocytes). J Natl Cancer Inst 54: 563-569.

Nishiyama Y and Rapp F (1981). Repair replication of viral and cellular DNA in Herpes simplex virus type 2-infected human embryonic and xeroderma pigmentosa cells. Virology 110: 466-475.

Noda T, Yajima H and Ito Y (1988). Progression of the phenotype of transformed cells after growth stimulation of cells by a human papillomavirus type 16 gene function. J Virol 62: 313-324.

Norval M, Howie S, Ross J and Maingay J (1986). The effect of uv-irradiation on viral infections of the skin. Microbiol Sci 3: 220-223.

Norval M and Bingham RW (1987). Advances in the use of nucleic acid probes in diagnosis of viral diseases of man. Arch Virol 97: 151-165.

Noyes W (1964). Structure of the human wart virus. Virology 23: 65-72.

Noyes WF and Mellors RC (1957). Fluorescent antibody detection of the antigens of the Shope papilloma virus in papilloma of the wild and domestic rabbit. J Exp Med 106: 555-561.

Nuovo GJ, Crum CP, De Villiers EM, Levine RH and Silverstein SJ (1988). Isolation of a novel human papillomavirus (type 51) from a cervical condyloma. J Virol 62: 1452-1455.

Obalek S, Glinski W, Haftek M, Orth G and Jablonska S (1980). comparative studies on cell-mediated immunity in patients with different warts. Dermatologica 161: 73-83.

Obalek S, Jablonska S and Orth G (1985). HPV-associated intra-epithelial neoplasia of external genitalia. Clin Dermatol 3: 104-113.

Obalek S, Favre M, Jablonska S, Szymanczyk J and Orth G (1988). Human papillomavirus type 2-associated basal cell carcinoma in two immunosuppressed patients. Arch Dermatol 124: 930-934.

Ogilvie MM (1970a). Virological and immunological aspects of infection with the human papova (wart) virus. M.D. Thesis, University of Edinburgh.

Ogilvie MM (1970b). Serological studies with human papova (wart) virus. J Hyg (Camb) 68: 479-490.

Oguchi M, Komura J, Tagami H and Ofuji S (1981a). Ultrastructural studies of spontaneously regressing plane warts: macrophages attack verruca-epidermal cells. Arch Dermatol Res 270: 403-411.

Oguchi M, Komura J, Tagami H and Ofuji S (1981b). Ultrastructural studies of spontaneously regressing plane warts: Langerhans cells show marked activation. Arch Dermatol Res 271: 55-61.

Olson C, Gordon DE, Robl MG and Lee KP (1969). Oncogenicity of bovine papilloma virus. Arch Environ Health 19: 827-837.

Olson C, Pamukcu AM and Brobst DF (1965). Papilloma-like virus from bovine urinary bladder tumours. Cancer Res 25: 840-850.

Oriel JD (1971a). Natural history of genital warts. Br J Vener Dis 47: 1-13.

Oriel JD (1971b). Anal warts and anal coitus. Brit J Vener Dis 47: 373-376.

Orth G (1986a). Epidermodysplasia verruciformis: a model for understanding the oncogenicity of human papillomaviruses. In "Papillomaviruses": CIBA Foundation Symp 120: 157-174.

Orth G (1986b). Discussion following "Human papillomavirus DNA in genital cancer" In "Papillomaviruses": CIBA Foundation Symp 120: 200.

Orth G, Breitburd F and Favre M (1978). Evidence for antigenic determinants shared by the structural polypeptides of (Shope) rabbit papillomavirus and human papillomavirus type 1. Virology 91: 243-253.

Orth G and Favre M (1985). Human papillomaviruses: biochemical and biologic properties. Clin Dermatol 3: 27-42.

Orth G, Favre M and Croissant O (1977). Charcterisation of a new type of human papillomavirus that causes skin warts. J Virol 24: 108-120.

Orth G, Favre M, Breitburd F, Croissant O, Jablonska S, Obalek S, Jarzabek-Chorzelska M and Rzesa G (1980). In: Viruses in Naturally Occurring Cancers. Ed. Essex M, Todaro G and zur Hausen H. Cold Spring Harbor (NY) Laboratory, 259-282.

Orth G, Jablonska S, Favre M, Croissant O, Obalek S, Jarzabek-Chorzelska M and Jibard N (1983). Identification of papillomaviruses in butcher's warts. J Invest Dermatol 76: 97-102.

Orth G, Jablonska S, Jarzabek-Chorzelska M, Obalek S, Rzesa G, Favre M and Croissant O (1979). Characteristics of the lesions and risk of malignant conversion associated with the type of human papillomavirus involved in epidermodysplasia verruciformis. Cancer Res 39: 1074-1082.

Orth G, Jeanteur P and Croissant O (1971). Evidence for and localisation of vegetative viral DNA replication by auto-radiographic detection of RNA-DNA hybrids in sections of tumours induced by Shope papilloma virus. Proc Natl Acad Sci USA 68: 1876-1880.

Ostrow RS, Bender M, Niimura M, Seki T, Kawashima M, Pass F and Faras AJ (1982). Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis. Proc Natl Acad Sci USA 79: 1634-1638.

Pardue ML (1985). In situ hybridisation in "Nucleic Acid Hybridisation" (Ed Hames BD and Higgins SJ) p179, IRL Press Oxford, 179-202.

Pass F, Janis R and Marcus DH (1971). Antigens of human wart tissue. J Invest Dermatol 56: 305-310.

Pass F and Maizel JV (1973). Wart-associated antigens. II. Human immunity to viral structural proteins. J Invest Dermatol 60: 307-311.

Pass F and Marcus DM (1973). Wart-associated antigens. I. Isolation of tissue antigens using antibody immunoadsorbents. J Invest Dermatol 60: 301-306.

Pass F, Reissig M, Shah KV, Eisinger M and Orth G (1977). Identification of an immunologically distinct papillomavirus from lesions of epidermodysplasia verruciformis. J Natl Cancer Inst 59: 1107-1112.

Patel KR, Smith KT and Campo MS (1987). The nucleotide sequence and genome organisation of bovine papillomavirus type 4. J Gen Virol 68: 2117-2128.

Patel R and Groff DB (1972). Condyloma accuminata in childhood. Paediatrics 50: 153-154.

Pater MM, Hughes GA, Hyslop DE, Nakshatri H and Pater A (1988). Glucocorticoid-dependent oncogenic tranformation by type 16 but not type 11 human papillomavirus DNA. Nature 335: 832-835.

Pater MM and Pater A (1985). Human papillomavirus types 16 and 18 sequences in carcinoma cell lines of the cervix. Virology 145: 313-318.

Pater MM and Pater A (1988). Expression of human papillomavirus types 16 and 18 DNA sequences in cervical carcinoma cell lines. J Med Virol 26: 185-195.

Paterson MEL, Peel KR and Joslin CAF (1984). Cervical smear histories of 500 women with invasive cervical cancer in Yorkshire. Br Med J 289: 896-898.

Patillo RA (1977). Tumour antigen and human chorionic gonadotropin in CaSki cells: a new epidermoid cervical cancer cell line. Science 196: 1456-1458.

Penn I (1975). The incidence of malignancies in transplant recipients. Transplant Proc 7: 323-326.

Penn I (1986). Cancer is a complication of severe immunosuppression. Surg Gynecol Obstet 162: 603-610.

Pfister H (1984). Biology and biochemistry of papillomaviruses. Rev Physiol Biochem Pharmacol 99: 112-181.

Pfister H (1987a). Current papillomavirus classification scheme. 6th International Papillomavirus Workshop: Short Review of Current Literature, 1-10.

Pfister H (1987b) Human papillomaviruses and genital cancer. Adv Cancer Res 48: 113-147.

Pfister H, Gassenmeier A, Nurnberger F and Stuttgen G (1983a). Human papillomavirus 5-DNA in a carcinoma of an epidermodysplasia verruciformis patient infected with various human papillomavirus types. Cancer Res 43: 1436-1441.

Pfister H, Gissmann L and zur Hausen H (1977). Partial characterisation of the proteins of human papillomaviruses (HPV) 1-3. Virology 83: 131-137.

Pfister H, Gross G and Hagedorn M (1979). Characterisation of human papillomavirus 3 in warts of a renal allograft patient. J Invest Dermatol 73: 349-353.

Pfister H, Hettlich I, Runne U, Gissmann L and Chilf GN (1983). Characterisation of human papillomavirus type 13 from focal epithelial hyperplasia Heck lesions. J Virol 47: 363-366.

Pfister H and zur Hausen H (1978). Seroepidemiological studies of human papillomavirus (HPV-1) infections. Int J Cancer 21: 161-165.

Phelps WC and Howley PM (1987). Transcriptional trans-activation by the human papillomavirus type 16 E2 gene product. J Virol 61: 1630-1638.

Phelps WC, Leary SL and Faras AJ (1985). Shope papillomavirus transcription in benign and malignant rabbit tumours. Virology 146: 120-129.

Phelps WC, Yee CL, Munger K and Howley PM (1988). The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of Adenovirus E1 A. Cell 53: 539-547.

Pilacinski WP, Glassman DL, Krzyzek RA, Sadowski PL and Robbins AK (1984). Cloning and expression in Escherichia coli of the bovine papillomavirus L1 and L2 open reading frames. Biotechnology 1: 356-360.

Pilotti S, Rilke F, Di Paolo G, Della Torre G and Alasio L (1981). Condylomata of the uterine cervix and koilocytosis of cervical and intraepithelial neoplasia. J Clin Pathol 34: 532-541.

Pirisi L, Yasumoto S, Feller M, Doniger J and Di Paolo JA (1987). Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J Virol 61: 1061-1066.

Popescu NC, Amsbaugh SC and Di Paolo J (1987). Human papillomavirus type 18 DNA is integrated at a single chromosome site in cervical carcinoma cell line SW 756. J Virol 61: 1682-1685.

Praetorius-Claussen F (1972). Rare oral viral disorders (molluscum contagiosum, localised keratoacanthoma, verrucae condyloma acuminatum and focal epithelial hyperplasia. Oral Surg 34: 604-618.

Prawer SE, Pass F, Vance JC, Greenberg LJ, Yunis EJ and Zelickson AS (1977). Depressed immune function in epidermodysplasia verruciformis. Arch Dermatol 113: 495-499.

Prentice RL and Thomas DB (1987). On the epidemiology of oral contraceptives and disease. Adv Cancer Res 49: 285-401.

Proniewska M and Jablonska S (1980). UV-induced DNA repair synthesis in patients with epidermodysplasia verruciformis. Dermatologica 60: 289-296.

Purola E and Savia E (1977). Cytology of gynecologic condyloma acuminatum. Acta Cytol 21: 26-31.

Pyrhonen S (1978). Human wart-virus antibodies in patients with genital and skin warts. Acta Dermatovenereol 58: 427-432.

Pyrhonen S and Johansson E (1975). Regression of warts - an immunological study. Lancet i: 592-596.

Pyrhonen S, Jablonska S, Obalek S and Kuismanen E (1980). Immune reactions in epidermodysplasia verruciformis. Br J Dermatol 102: 247-253.

Quick CA, Faras A and Krzysek R (1978). The etiology of laryngeal papillomatosis. The Laryngoscope 88: 1789-1795.

Rando RF, Lancaster WD, Han P and Lopez C (1986a). The noncoding region of the HPV-6vc contains two distinct transcriptional enhancing elements. Virology 155: 545-566.

Rando RF, Sedlacek TV, Hunt J, Bennett Jenson A, Kurman RJ and Lancaster WD (1986b). Verrucous carcinoma of the vulva associated with an unusual type 6 human papillomavirus. Obstet Gynecol 67: 70s-75s.

Ravetch JV, Luster AB, Weinshank R, Kochan J, Pavlovec A, Portnoy DA, Hulmes J, Pan Y-CE and Unkeless JC (1986). Structural heterogeneity and functional domains of murine immunoglobulin G Fc receptors. Science 234: 718-725.

Rawls WE, Adam E and Melnick JL (1973). An analysis of seroepidemiological studies of herpesvirus type 2 and carcinoma of the cervix. Cancer Res 33: 1477-1482.

Rebello R, Green FNY and Fox H (1975). A study of the secretory immune system of the female genital tract. Br J Obstet Gynaecol 82: 812-816.

Reeves WC, Caussy D, Brinton LA, Brenes MM, Montalyan P, Gomez B, De Britton RC, Morice E, Gaitan E, Loo de Lao S and Rawls WE (1987). Case control study of human papillomaviruses and cervical cancer in Latin America. Int J Cancer 40: 450-454.

Reid R, Fu YS, Herschman BR, Crum CP, Braun L, Shaw KV, Agronow SJ and Stanhope CR (1984). Genital warts and cervical cancer. VI. The relationship between aneuploid and polyploid cervical lesions. Am J Obstet Gynecol 150: 189-199.

Reid R, Laverty CR, Coppleson M, Isarangkul W and Hills E (1980). Non-condylomatous cervical wart virus infection. Obstet Gynecol 55: 476-483.

Reid R, Stanhope R, Herschman BR, Booth E, Phibbs GD and Smith JP (1982). Genital warts and cervical cancer. I. Evidence of association between subclinical papillomavirus infection and cervical malignancy. Cancer 50: 377-387.

Reilly SS and Taichman LB (1987). Under-replication of human papillomavirus type-1 DNA in cultures of foreskin keratinocytes. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 159-164.

Richman DD, Cleveland PH, Redfield DC, Oxman MN and Wahl GM (1984). Rapid Viral Diagnosis. J Infec Dis 149: 298-310.

Rigby PHJ, Dieckmann M, Rhodes C and Berg P (1977). Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113: 237-251.

Rigoni-Stern (1842). Fatti statistici relativi alle malatie cancerose. Gior Servire Progr Path Terap 2: 507-517.

Riou GF, Barrdis M, Dutronquay V and Orth G (1985). Presence of papillomavirus DNA sequences, amplification of c-myc and c-Ha-ras oncogenes and enhanced expression of c-myc in carcinomas of the uterine cervix. UCLA Symp. Mol. Cell. Biol. New Series 32: 47-56.

Robertson DAF, Guy EC, Hang SL and Wright R (1987). Persistent measles genome in autoimmune chronic active hepatitis. Lancet ii: 9-11.

Roitt IM, Brostoff J and Male DK (1985). Immunology, published by Churchill Livingstone, Edinburgh.

Roman A and Fife K (1986). Human papillomavirus DNA associated with foreskins of normal newborns. J Infect Dis 153: 855-861.

Rose BR, Thompson CH, McDonald AM, Henderson BR, Cossart YE and Morris BJ (1987). Cell biology of cultured anogenital warts. Br J Dermatol 116: 311-322.

Rothbard JB and Taylor WR (1988). A sequence pattern common to T cell epitopes. EMBO J 7: 93-100.

Rotkin JD (1973). A comparison review of key epidemiological studies in cervical cancer related to current searches for transmissble agents. Cancer Res 33: 1353-1367.

Rous P and Friedewald (1941). The effect of chemical carcinogens on virus-induced rabbit carcinomas. J Exp Med 79: 511-537.

Rowson KEK and Mahy BWJ (1967). Humam papova (wart) virus. Bact Rev 31: 110-131.

Roychoudhury R, Jay E and Wu R (1976). Terminal labelling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. Nucleic Acid Res 3: 863-877.

Rudlinger R, Bunney MH, Grob R and Hunter JAA (1989). Warts in Fish handlers. Br J Dermatol 120: 375-381.

Rudlinger R, Smith IW, Bunney MH and Hunter JAA (1986). Human papillomavirus infections in a group of renal transplant recipients. Br J Dermatol 115: 681-692.

Ruiter M and Mullem PJ van (1966). Demonstration of electron-microscopy of an intranuclear virus in epidermodysplasia verrucuiformis. J Invest Dermatol 47: 247-252.

Ruiter M and Mullem PJ van (1970). Behaviour of virus in malignant degeneration of skin lesion in epidermodysplasia verruciformis. J Invest Dermatol 54: 324-331.

Sadoul G and Beuret T (1984). Treatment of cervical and vulvar condylomata with CO_2 laser combined with an immunostimulant. Rev. Fr. Gynecol Obstet 79: 681-684.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA and Arnheim N (1985). Enzymatic amplification of ∝-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science 230: 1350-1354.

Sano T, Kudo H, Sugimoto T and Shikata E (1988). Synthetic oligonucleotide hybridisation probes to diagnose hop stunt viroid strains and citrus exocortis viroid. J Virol Methods 19: 109-120.

Sasson IM, Haley NJ, Hoffmann D, Wylander, EL, Hellberg D and Nilsson S (1985). Cigarette smoking and neoplasia of the uterine cervix: smoke constituents in cervical mucous. New Eng J Med 312: 315-316.

Sauder DN (1985). Biologic properties of epidermal cell thymocyte-activating factor (ETAF). J Invest Dermatol 85: 176S-179S.

Schiller JT (1987). Papillomavirus genes: Function and protein products. 6th International Papilloma Workshop. Short Review of current literature 43-51.

Schiller JT, Vass WC and Lowy DR (1984). Identification of a second transforming region in bovine papillomavirus DNA. Proc Natl Acad Sci USA 81: 7880-7884.

Schiller JT, Vass WC, Vousden KH and Lowy DR (1986). E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J Virol 57: 1-6.

Schlegel R, Wade-Glass M, Rabson MS and Yang Y-C (1986). The E5 transforming gene of bovine papillomavirus encodes a small hydrophobic polypeptide. Science 233: 464-467.

Schlegel R and Wade-Glass M (1987). E5 transforming polypeptide of bovine papillomavirus. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 87-91.

Schneider A, Kraus H, Schumann R and Gissmann L (1985). Papillomavirus infection of the lower genital tract: Detection of viral DNA in gynecological swabs. Int J Cancer 35: 443-448.

Schneider-Gadicke A and Schwarz E (1986). Different human cervical carcinoma cell lines show similar transcription patterns of human papilloma type 18 early genes. EMBO J 5: 2285-2292

Schneider-Maunoury S, Georges E and Orth G (1987). Structure and expression of the cottontail rabbit papillomavirus genome and of myc and cis genes during the progression of domestic rabbit skin tumours. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 229-236.

Schneider A, Sawada E, Gissmann L and Shah K (1987). Human papillomaviruses in women with a history of abnormal papanicolaou smears and in their male partners. Obstet Gynecol 69: 554-562.

Schwartz E, Durst M, Demankowski C, Latterman O, Zech R, Wolfsberger E, Suhai S and zur Hausen H (1983). DNA sequence and genome organisation of genital human papillomavirus type 6b. EMBO J 2: 2341-2348.

Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau, A and zur Hausen H (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 314: 111-114.

Schwarz E and Schneider-Gadicke A (1986). Expression of human papillomavirus type-18 DNA in cervical carcinoma cell lines. In DNA Tumor Viruses: Control and Gene Expression and Replication (Ed Botchan M, Grodzicker T and Sharp PA) Cancer Cells 4: Cold Spring Harbor (NY) Laboratory, 581-587.

Schwarz E, Schneider-Gadicke A and zur Hausen H (1987). Human papillomavirus type-18 transcription in cervical carcinoma cell lines and in human cell hybrids. In Papillomaviruses (Ed Steinberg BM, Branndsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 47-53.

Scott GM and Csonka GW (1979). Effect of infections of small doses of human fibroblast interferon into genital warts. Br J Vener Dis 55: 442-445.

Seedorf K, Krammer G, Durst M, Suhai S and Rowekamp WG (1985). Human papillomavirus type 16 DNA sequence. Virology 145: 181-185.

Seedorf K, Oltersdorf T, Krammer G and Rowekamp WG (1987). Identification of early proteins of the human papillomavirus type 16 (HPV-16) and type 18 (HPV-18) in cervical carcinoma cells. EMBO J 6: 139-144.

Seski JC, Reinhalter ER and Silva J (1978). Abnormalities of lymphocyte transformations in women with condylomata accuminata. Obstet Gynecol 51: 188-192.

Seto A, Alehi SA, Miyanomae K, Ushijima T and Ito Y (1980). Anaerobic coryneform bacteria as normal flora of rabbit skin and regression of Shope papillomas. Oncology 37: 12-15.

Sheridan JF, Beck M, Aurelian L and Radowsky M (1985). Immunity to herpes simplex virus: virus reactivation modulates lymphokine activity. J Infect Dis 152: 449-456.

Sheridan JF, Beck M, Smith CC and Aurelian L (1987). Reactivation of herpes simplex virus is associated with production of a low molecular weight factor that inhibits lymphokine activity in vitro. J Immunol 138: 1234-1239.

Sheridan JF, Donnenberg AD, Aurelian L and Elpern DJ (1982). Immunity to herpes simplex virus type 2. IV. Impaired lymphokine production during recrudescence correlates with an imbalance in T lymphocyte subsets. J Immunol 129: 326-331.

Shibata DK, Arnheim N and Martin WJ (1988). Detection of human papillomavirus in paraffin-embedded tissue using the polymerase chain reaction. J Exp Med 167: 225-230.

Shirasawa H, Tomita Y, Kubota K, Kasai T, Sekiya S, Takamizawa H and Simizu B (1986). Detection of human papillomavirus type 16 DNA and evidence for integration into the cell DNA in cervical dysplasia. J Gen Virol 67: 2011-2015.

Shirasawa H, Tomita Y, Sekiya S, Takamizawa H and Simizu B (1987). Integration and transcription of human papillomavirus type 16 and 18 sequences in cell lines derived from cervical carcinomas. J Gen Virol 68: 583-591.

Shirodaria PV and Matthews RS (1975). An immunofluorescence study of warts. Clin Exp Immunol 21: 329-338.

Shope RE and Hurst EW (1933). Infectious papillomatosis of rabbits; with a note on the histopathology. J Exp Med 58: 607-624

Sillman FH and Sedlis A (1987). Anogenital papillomavirus infection neoplasia in immunodeficient women. Obstet Gynecol Clin North Am 14: 537-558.

Singer A, Reid BL and Coppleson M (1976). A hypothesis: the role of a high-risk male in the aetiology of cervical carcinoma. A correlation of epidemiology and molecular biology. Am J Obstet Gynecol 126: 110-115.

Singer A and McCance D (1985). The wart virus and genital neoplasia: a casual or causal association. Br J Obstet Gynaecol 92: 1083-1085.

Skegg DCG, Corwin PA, Paul C and Doll R (1982). Importance of the male factor in cancer of the cervix. Lancet i: 581-583.

Smith HO and Wilcox KW (1970). a restriction enzyme from Haemophilus influenzae I. Purification and general properties. J Mol Biol 51: 379-391.

Smith KT, Campo MS, Bradley J, Gaukrodger J and Jarrett WFH (1987). Cell transformation by bovine papillomavirus: cofactors and cellular responses. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 267-274.

Smith KT and Campo MS (1988). "Hit and Run" transformation of mouse C127 cells by bovine papillomavirus type 4: the viral DNA is required for the initiation but not for maintenance of the transformed phenotype. Virology 164: 39-47.

Smotkin D and Wettstein FO (1986). Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc Natl Acad Sci USA 83: 4680-4684.

Smotkin D and Wettstein FO (1987). The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J Virol 61: 1686-1689.

Sontheimer RD, Bergstresser PR, Gailiunas P, Helderman JH and Gilliam JN (1984). Perturbation of epidermal Langerhans cells in immunosuppressed human renal allograft recipients. Transplantation 37: 168-174.

Southern EM (1975). Detection of specific sequences among DNA fragments separated by Gel electrophoresis. J Mol Biol 98: 503-517.

Spalholz BA, Yang Y-C and Howley PM (1985). Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. Cell 42: 183-191.

Spencer ES and Andersen HK (1970). Clinically evident, non-terminal infections with herpesviruses and the wart virus in immunosuppressed renal allograft recipients. Br Med J iii: 251-254.

Spradbrow PB, Beardmore GL and Francis J (1983). Virions resembling papillomaviruses in hyperkeratotic lesions from sun-damaged skin. Lancet i: 189.

Steele K, Shirodaria PV, Pfister H, Pollock B, Fuchs B, Merrett JD, Irwin WG and Simpson DIH (1988). A study of HPV-1,2 and 4 antibody prevalence in patients presenting for treatment with cutaneous warts to general practitioners in N. Ireland. Epidemiol Infect 101: 537-546.

Steinberg BM and Abrahamson AL (1985). Laryngeal papillomas. Clin in Dermatol 3: 130-138.

Steinberg BM, Abramson AL and Meade R (1982). Culture of human laryngeal papilloma cells in vitro. Otolaryngol - Head Neck Surg 90: 728-735.

Steinberg BM, Topp WC, Schneider PS and Abramsom AL (1983). Laryngeal papillomavirus infection during clinical remission. New Eng J Med 308: 1261-1264.

Stevens JG and Wettstein FO (1979). Multiple copies of Shope virus DNA are present in cells of benign and malignant non-virus-producing neoplasms. J Virol 30: 891-898.

Stingl G, Wolff-Schreiner EC, Pichler WJ, Gschnait F and Knapp W (1977). Epidermal Langerhans cells bear Fc and C3 receptors. Nature 268: 245-246.

Stingl G, Katz SI, Clement L, Green I and Shevach EM (1978). Immunologic functions of Ia-bearing epidermal Langerhans cells. J Immunol 121: 2005-2013.

Stoler MH and Broker TR (1986). In situ hybridisation of human papilloma DNAs and messenger RNAs in genital condylomas and a cervical carcinoma. Human Pathol 17: 1250-1258.

Stone RS, Shope RE and Moore DH (1959). Electron microscope study of the development of the papillomavirus in the skin of the rabbit. J Exp Med 110: 543-546.

Storey A, Pim D, Murray A, Osborn K, Banks L and Crawford L (1988). Comparison of the in vitro transforming activities of human papillomaviruses types. EMBO J 7: 1815-1820.

Strauss NJ, Bunting H and Melnick JL (1950). Virus-like particles and inclusion bodies in skin papillomas. J Invest Dermatol 15: 433-444.

Streilein JW and Bergstresser PR (1980). Ia antigens and epidermal Langerhans cells. Transplantation 30: 319-323.

Stremlau A, Gissmann L, Ikenberg H, Stark M, Bannasch P and zur Hausen H (1985). Human papillomavirus type 16 related DNA in an anaplastic carcinoma of the lung. Cancer 55: 1737-1740.

Strike D, Rose R, Mattison HR and Reichman R (1986). Cloning and expression of HPV 6b capsid peptides: localisation of an immunoreactive region. J Cell Biochem Suppl. 220.

Subak-SharpeJH, Burk RR, Crawford LV, Morrison JM, Hay J and Keir HM (1966). An approach to evolutionary relationships of mammalian DNA viruses through analysis of the pattern of nearest neighbour base sequence. Cold Spring Harbor Symp Quant Biol 31: 737-748.

Sugawara K, Fujinaga K, Yamashita T and Ito Y (1983). Integration and methylation of Shope papilloma virus DNA in the transplantable Vx2 and Vx7 rabbit carcinomas. Virology 131: 88-99.

Sundberg JP, O'Banion MK and Reichmann ME (1987). Mouse papillomavirus: pathology and characterisation of the virus. In Papillomaviruses (Ed Steinberg BM, Bransdma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 373-379.

Syrjanen K (1983a). Human papillomavirus lesions in association with cervical dysplasias and neoplasias. Obstet Gynaecol 62: 617-624.

Syrjanen K (1983b). Immunocompetent cells in uterine cervical lesions of human papillomavirus origin. Gynecol Obstet Invest 16: 327-340.

Syrjanen K, De Villiers EM, Saarikoski S, Castren O, Vayrynen M, Mantyjarvi R and Parkkinen S (1985a). Cervical papillomavirus infection progressing to invasive cancer in less than three years. Lancet i: 510-511.

Syrjanen K, Mantyjarvi R, Saarikoski S, Varynen M, Syrjanen S, Parkkinen S, Yliskoski M, Saastamoinen J and Castren O (1988). Factors associated with progression of cervical human papillomavirus (HPV) infections into carcinoma in situ during a long-term prospective follow-up. Br J Obstet Gynaecol 95: 1096-1102.

Syrjanen K, Mantyjarvi R, Vayrynan M, Syrjanen S, Parkkinen S, Yliskoski M, Saarikoski S, Sarkkinen H, Nurmi T and Castren O (1987a). Assessing the biological potential of human papillomavirus infections in cervical carcinogenesis. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 281-289.

Syrjanen S, Partanen P and Syrjanen K (1987b). Comparison of *in situ* DNA hybridisation protocols using ^{35}S-labelled and biotin-labelled probes in detection of human papillomavirus DNA sequences. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5: Cold Spring Harbor (NY) Laboratory, 329-336.

Syrjanen K, Vayrynen M, Castren O, Mantyjarvi R and Yliskoski M (1984). The relation between the type of immunoreactive cells found in human papillomavirus (HPV) lesions of the uterine cervix and the subsequent behaviour of these lesions. Arch Gynecol 234: 189-196.

Syrjanen K, Vayrynan M, Saarikoski S, Mantyjarvi R, Parkkinen S, Hippelainen M and Castren O (1985b). Natural history of cervical human papillomavirus (HPV) infections based on prospective follow-up. Br J Obstet Gynecol 92: 1086-1982.

Tagami H, Ogino A, Takigawa M, Imamura S and Ofuji S (1974). Regression of plane warts following spontaenous inflammation. Br J Dermatol 90: 147-154.

Tagami H, Oku T and Iwatsuki K (1985). Primary tissue culture of spontaneously regressing flat warts. *In vitro* attack by mononuclear cells against wart-derived epidermal cells. Cancer 55: 2437-2441.

Tagami H, Takigawa M, Ogino A, Imamura S and Ofuji S (1977). Spontaneous regression of plane warts after inflammation. Arch Dermatol 113: 1209-1213.

Taichman LB, Breitburd F, Croissant O and Orth G (1984). The search for a culture system for papillomavirus. J Invest Dermatol 83: 2S-6S.

Tay SK, Jenkins D, Maddox P, Campion M and Singer A (1987a). Subpopulations of Langerhans cells in cervical neoplasia. Br J Obstet Gynecol 94: 10-15.

Tay SK, Jenkins D, Maddox P and Singer A (1987b). Lymphocyte phenotypes in cervical intraepithelial neoplasia and human papillomavirus infection. Br J Obstet Gynecol 94: 16-21.

Tay SK, Jenkins D and Singer A (1987c). Natural killer cells in cervical intraepithelial neoplasia and human papillomavirus infection. Br J Obstet Gynecol 94: 901-906.

Tay S, Jenkins D, Maddox P, Hogg N and Singer A (1987d). Tissue macrophage response in human papilloma infection and cervical intraepithelial neoplasia. Br J Obstet Gynecol 94: 1094-1097.

Ten Berge RJM, Schellekens PTA, Surachno S, The TH, Ten Veen JH and Wilmink JM (1981). The influence of therapy with azothioprine and prednisone on the immune system of kidney transplant patients. Clin Immunol Immunopathol 21: 20-32.

Terry RM, Lewis FA, Griffiths S, Wells M and Bird CC (1987). Demonstration of human papillomavirus types 6 and 11 in juvenile laryngeal papillomatosis by *in situ* DNA hybridisation. J Pathol 153: 245-248.

Thivolet J, Hegazy MR, Viac J and Chardonnet Y (1977). An *in vitro* study of cell mediated immunity in human warts. Acta Dermatol Venereol 57: 317-319.

Thomas M, Boiron M, Tanzer J, Levy JP and Bernard J (1964). *In vitro* transformation of mice cells by bovine papillomavirus. Nature 202: 709-710.

Tidy JA, Parry GCN, Ward P, Coleman DV, Peto J, Malcolm ADB and Farrell PJ (1989). High rate of human papillomavirus type 16 infection in cytologically normal cervices. Lancet i: 434.

Tilbrook PA, Greenoak GE, Reeve VE, Canfield PJ, Gissmann L, Gallagher CH and Kulski JK (1989). Identification of papillomaviral DNA sequences in hairless mouse tumours induced by ultraviolet irradiation. J Gen Virol 70: 1005-1009.

Townsend ARM, Rothbard J, Gotch FM, Bahadur G, Wraith D and McMichael AJ (1986). The epitopes of influenza nucleoprotein recognised by cytotoxic T lymphocytes can be defined into short synthetic peptides. Cell 44: 959-968.

Trevathan E, Layde P, Webster LA, Adams JB, Benigno BB and Ory H (1983). Cigarette smoking and dysplasia and carcinoma in situ of the uterine cervix. J Am Med Assoc 250: 499-500.

Toews GB, Bergstresser PR, Streilein JW and Sullivan S (1980). Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. J Immunol 124: 445-453.

Tong MJ, Wallace AM, Peters RL and Reynolds TB (1975). Lymphocyte stimulation in Hepatitis B infections. New Eng J Med 293: 318-322.

Tourtellotte WN, Verity AN, Schmid P, Martinez S and Shapshak P (1987). Covalent binding of formalin fixed paraffin embedded brain tissue sections to glass slides suitable for in situ hybridisation. J Virol methods 15: 87-99.

Underdown BJ and Schiff JM (1986). Immunoglobulin A: strategic defence initiative at the mucosal surface. Ann Rev Immunol 4: 389-417.

Ungar ER, Budgeon LR, Myerson D and Brigati DJ (1986). Viral diagnosis by in situ hybridisation. Description of a rapid simplified colormetric method. Am J Surg Pathol 10: 1-8.

Urdea MS, Warner BD, Running JA, Stempion M, Clyne J and Horn T (1988). A comparison of non-radioisotopic hybridisation assay methods using fluorescent, chemiluminescent and enzyme labelled synthetic oligodeoxyribonucleic probes. Nucleic Acid Res: 16: 4937-4956.

Van der Leest RJ, Zachow KR, Ostrow RS, Bender M, Pass F and Faras AJ (1987). Human papillomavirus heterogeneity in 36 renal transplant recipients. Arch Dermatol 123: 354-357.

Vanselow BA, Spradbrow PB and Jackson ARB (1982). Papillomaviruses, papillomas and squamous cell carcinomas in sheep. Vet Rec 110: 561-562.

Vessey MP, McPherson K, Lawless M and Yeates D (1983). Neoplasia of the cervix uteri and contraception: a possible adverse effect of the pill. Lancet ii: 930-934.

Vestey JP, Howie SEM, Norval M, Maingay JP and Neill WA (1988a). Immune responses to herpes simplex virus in patients with facial herpes simplex and those with eczema herpeticum. Br J Dermatol 118: 775-782.

Vestey JP, Howie SEM, Norval M, Maingay JP and Hunter JAA (1988b). Epidermal antigen presenting cells in patients with facial herpes simplex virus infections. in "The Langerhans Cell" Ed. Thivolet J and Schmitt D, Colloque Inserm 172: 343.

Viac J, Staquet MJ, Miquet M, Chabanon M and Thivolet J (1978). Specific immunity to human papilloma virus (HPV) in patients with genital warts. Br J Vener Dis 54: 172-175.

Viac J, Thivolet J and Chardonnet Y (1977a). Specific immunity in patients suffering from recurring warts before and after repetitive intradermal tests with human papillomavirus. Br J Dermatol 97: 365-370.

Viac J, Thivolet J, Hegazy VR, Chardonnet Y and Dambuyant C (1977b). Comparative study of delayed hypersensitivity skin reactions and antibodies to human papillomavirus (HPV). Clin Exp Immunol 29: 240-246.

Vinograd J, Lebowitz J, Radloff R, Watson R and Laipis P (1965). The twisted circular form of polyoma viral DNA. Proc Natl Acad Sci USA 53: 1104-1111.

Voller A and Bidwell DE (1975). A simple method for detecting antibodies to rubella. Br J Exp Path 56: 338-339.

Von Knebel Doeberitz C, Bunge R, Buchert J and De Villiers E-M (1988) Human papillomaviruses: Compilation of restriction endonuclease analyses. Personal Communication.

Waelsch L (1918). Ubertagungsversuche mit spitzen kondylom. Arch Dermatol Syphilis 124: 625-646.

Wagner D, Ikenberg H, Boehm N and Gissmann L (1984). Identification of human papillomavirus in cervical swabs by deoxyribonucleic acid in situ hybridisation. Obstet Gynecol 64: 767-772.

Walker PG, Singer A, Dyson JL, Shah KV, To A and Coleman DV (1983). The prevalence of human papillomavirus antigen in patients with cervical intraepithelial neoplasia. Br J Cancer 48: 99-101.

Watanabe S, Soeda E, Uchida S and Yoshike K (1984). DNA rearrangement affecting expression of the BK virus transforming gene. J Virol 51: 1-6.

Watson JD and Littlefield JW (1960). Some properties of DNA from Shope papillomavirus. J Mol Biol 2: 161-165.

Watson J, Mochizuki D and Gillis S (1983). T-cell growth factors: interleukin 2. In : "T Lymphocytes Today" Ed. JR Inglis, Elsevier Science Publishers, 96-99.

Watts S, Phelps WC, Ostrow RS, Zachow KR and Faras AJ (1984). Cellular transformation by human papillomavirus DNA in vitro. Science 225: 634-636.

Wettstein FO and Stevens JG (1981). Transcription of the viral genome in papillomas and carcinomas induced by the Shope virus. Virology 109: 448-451.

Wettstein FO and Stevens JG (1982). Variable sized free episomes of Shope papillomavirus DNA are present in all non-virus producing neoplasms and integrated episomes are detected in some. Proc Natl Acad Sci (USA) 79: 790-794.

Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA and Harlow E (1988). Association between an oncogene and and anti-oncogene: the adenovirus E1 A proteins bind to the retinoblastoma gene product. Nature 334: 124-129.

Wickenden C, Coleman DV, Evans B and Malcolm ADB (1987). Cross-hybridisation of human papillomavirus DNA on filters. J Virol Methods 15: 249-256.

Wickenden C, Steele A, Malcolm ADB and Coleman DV (1985). Screening for wart virus infection in normal and abnormal cervices by DNA hybridisation of cervical scrapes. Lancet i: 65-67.

Wildy P, Field WJ and Nash AA (1982). Classical herpes latency revisited. In : "Virus Persistence" 33rd Symposium of the Society for General Microbiology. (Ed. Mahy BWJ, Minson AC and Darby GK), 133-167.

Wilton JMA, Ivanyi L and Lehner T (1972). Cell-mediated immunity in herpesvirus hominius infections. Br Med J i: 723-726.

Winkler B, Krumholz B, Galli R, Nizet V, Palefsky J, Schoolnik G and Steinberg B (1986). Multifocal lower female genital tract condyloma/neoplasia syndrome: A clinico-pathologic and molecular analysis. J Cell Biochem 10C: 221.

Winkelstein W (1977). Smoking and cancer of the uterine cervix: hypothesis. Am J Epidemiol 106: 257-259.

Witkin SS (1987). Transient, local immunosuppression in recurrent vaginitis. Immunol Today 8: 360.

Wolff K and Winkelmann RK (1967). Ultrastructural localisation of nucleoside triphosphatase in Langerhans cells. J Invest Dermatol 48: 50-54.

Wood GS and Warnke R (1981). Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. J Histochem Cytochem 29: 1196-1204.

Woodruff JD, Braun L, Cavalieri R, Gupta P, Pass F and Shah KV (1980). Immunologic identification of papillomavirus antigen in condyloma tissues from the female genital tract. Obstet Gynecol 56: 727-732.

Woodworth CD, Doniger J and Di Paolo JA (1989). Immortalisation of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. J Virol 63: 159-164.

Yabe Y, Sadakane H and Isono H (1979). Connection between capsomeres in human papillomavirus. Virology 96: 547-552.

Yang Y-C, Okayama H and Howley PM (1985). Bovine papillomavirus contains multiple transforming genes. Proc Natl Acad Sci USA 82: 1030-1034.

Yaniv M (1986). Discussion following "Production of spliced DNA copies of the cottontail rabbit papillomavirus genome in a retroviral vector" by Danos O, Mulligan RC and Yanif M. In "Papillomaviruses": CIBA Foundation Symposium 120: 81.

Yasumoto S, Burkhardt AL, Doniger J and Di Paolo JA (1986). Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J Virol 57: 572-577.

Yee C, Krishnan-Hewlett I, Baker CC, Schlegel R and Howley DM (1985). Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am J Pathol 117: 361-366.

Young LS, Bevan IS, Johnson MA, Blomfield PI, Bromidge T, Maitland NJ and Woodman CBJ (1989). The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. Br Med J 298: 14-18.

Yutsudo M, Okamoto V and Hakura A (1988). Functional dissociation of transforming genes of human papillomavirus type 16. Virology 166: 594-597.

Yutsudo M, Shimakage T and Hakura A (1985). Human papillomavirus type 17 DNA in skin carcinoma tissue of a patient with epidermodysplasia. Virology 144: 295-298.

Zachow KR, Ostrow RS and Faras AJ (1987). Nucleotide sequence and genome organisation of human papillomavirus type 5. Virology 158: 251-254.

Zaninetti P, Franceschi S, Baccolo B, Bonazzi B, Gottardi G and Serraino D (1986). Characterisatics of women under 20 with cervical intraepithelial neoplasia. Int J Epidemiol 15: 477-482.

Zinkernagel RM, Hengartner H and Stitz L (1985). On the role of viruses in the evolution of immune responses. Br Med Bull 41: 92-97.

Zunzunegui MV, King M-L, Coria CF and Charlet J (1986). Male influences on cervical cancer risk. Am J Epidemiol 123: 302-307.

zur Hausen H (1976). Condylomata accuminata and human genital cancer. Cancer Res 36: 794.

zur Hausen H (1977). Human papillomaviruses and their possible role in squamous cell carcinomas. Curr Top Microbiol Immunol 78: 1-30.

zur Hausen H (1980). The role of viruses in human tumours. Adv Cancer Res 33: 77-107.

zur Hausen H (1982). Human genital cancer: synergism between two virus infections or synergism vetween a virus infection and initiating events. Lancet ii: 1380-1372.

zur Hausen H (1986). Intracellular surveillance of persisting viral infections. Human genital cancer results from deficient cellular control of papillomavirus gene expression. Lancet ii: 489-491.

zur Hausen H, Meinhof W, Scheiber W and Bernkamm GW (1974). Attempts to detect virus-specific DNA in human tumours. I. Nucleic acid hybridisation with complementary RNA of human wart virus. Int J Cancer 13: 650-656.

ADDENDUM

Alexander ER (1973). Possible etiologies of cancer of the cervix other than herpesvirus. Cancer Res 33 : 1485-1496.

Almeida JD, Oriel JD and Stannard LM (1969). Characterisation of the virus found in human genital warts. Microbios 3 : 225-232.

Burns J, Chan VTW, Jonasson JA, Fleming KA, Taylor S and McGee JO'D (1985). Sensitive system for visualising biotinylated DNA probes hybridised in situ : rapid sex determination of intact cells. J Clin Pathol 38: 1085-1092.

Coghlan JP, Aldred P, Haralambidis J, Niall HD, Penschow JD and Tregear GW (1985). Hybridisation histochemistry. Analytical Biochem. 149 : 1-28.

De Villiers EM, Wagner D, Schneider A, Wesch H, Miklaw H, Wahrendorf J, Papendick U and zur Hausen H (1987). Human papilloma-virus infections in women with and without abnormal cervical cytology. Lancet ii : 703-705.

Giri I, Danos O and Yaniv M (1985). Genomic structure of the cottontail rabbit (Shope) papillomavirus. Proc Natl Acad Sci USA, 82 : 1580-1584.

Haugen TH, Cripe TP and Turek LP (1987). An E2 gene product of bovine papillomavirus type-1 *trans* activates the upstream viral early gene promoter, P_{89}. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5 : Cold Spring Harbor (NY) Laboratory, 39-46.

Neill WA and Miller EP (1987). Generation of cytotoxic T cells to cytomegalovirus in patients with cervical intraepithelial neoplasia. Med Lab Sci 44 : 141-149.

Rosl F, Durst M and zur Hausen H (1988). Selective suppression of human papillomavirus transcription in non-tumorigenic cells by 5-azacytidine. EMBO J 7 : 1321-1328.

Schwarz E, Schneider-Gadicke A and zur Hausen H (1987). Human papillomavirus type-18 transcription in cervical carcinoma cell lines and in human cell hybrids. In Papillomaviruses (Ed Steinberg BM, Brandsma JL and Taichman LB) Cancer Cells 5 : Cold Spring Harbor (NY) Laboratory, 47-53.

Vonka V, Kanka J, Hirsch I, Zavadova H, Krcmar M, Suchankova A, Rezacova D, Broucek J, Press M, Domorazkova E, Svoboda B, Havrankova A and Jelinek J (1984). Prospective study on the relationship between cervical neoplasia and herpes simplex type-2 virus II. Herpes simplex type-2 antibody presence in sera taken at enrolment. Int J Cancer 33 : 61-66.

Wettstein FO, Barbosa MS and Nasseri M (1987). Identification of the major cottontail rabbit papillomavirus late RNA cap site and mapping and quantitation of an E2 and minor E6 coding mRNA in papillomas and carcinomas. Virology 159 : 321-328.

Yamasaki E and Ames BN (1977). Concentration of mutagens from urine by adsorption with the nonpolar resin XAD-2 : cigarette smokers have mutagenic urine. Proc Natl Acad Sci USA 74 : 3555-3559.

APPENDIX - PUBLISHED PAPERS

PUBLISHED PAPERS

Cubie H.A., and Norval M. (1988). Humoral and cellular immunity to papillomavirus in patients with cervical dysplasia. J Med Virol 24 : 85-95.

Cubie H.A., and Norval M. (1988). Synthetic oligonucleotide probes for the detection of human papilloma viruses by in situ hybridisation. J Virol Methods 20 : 239-249.

Cubie H.A., and Norval M. The detection of human papillomaviruses in paraffin sections with biotinylated synthetic oligonucleotide probes and immunogold staining. J Clin Pathol (in press).

Journal of Medical Virology 24:85–95 (1988)

Humoral and Cellular Immunity to Papillomavirus in Patients With Cervical Dysplasia

Heather A. Cubie and Mary Norval

Department of Bacteriology, University of Edinburgh Medical School, Edinburgh EH8 9AG, United Kingdom

The cell-mediated and humoral immune responses to human papillomavirus (HPV) were tested in groups of patients with various degrees of cervical intraepithelial neoplasia (CIN) using a lymphocyte proliferation assay (LPA) as a measure of circulating sensitised T-cells and an enzyme-linked immunosorbent assay (ELISA) for antibodies.

Twenty-three of 92 patients (25%) gave stimulation indices (S.I.) greater than two to at least one of the several antigen preparations tested in the LPA. Of 282 patients, 144 (50.1%) showed ELISA indices (E.I.) greater than one to HPV-1 and/or HPV-2 antigens prepared by disruption of purified virions.

No correlation was found between positive responses in either test and the presence in cervical biopsies of koilocytes (considered pathognomonic for HPV infections), or between positive responses and the degree of dysplasia observed. Rather, positive antibody and T-cell responses corresponded with a history of past or present skin warts. Although antibody was detected in 42/86 (48.8%) women who thought they had never had warts, only 2/24 (8.3%) with no known history gave a positive S.I. in LPA.

Key words: human papilloma virus, cell mediated immunity, lymphocyte proliferation assay, humoral immunity, ELISA, cervical intraepithelial neoplasia

INTRODUCTION

The ballooned cells with distorted nuclei and large halos found in cervical dysplasia and termed "koilocytes" [Koss and Durfee, 1956] were shown by Meisels et al [1977] to contain human papilloma virus (HPV) by electron microscopy and immunocytochemistry. This was confirmed by Hills and Laverty in 1979, and since then, several types of HPV have been identified in such lesions; most commonly, types 6 and 11 in cervical

Accepted for publication April 10, 1987.

Address reprint requests to Heather A. Cubie, Department of Bacteriology, University of Edinburgh Medical School, Edinburgh EH8 9AG, United Kingdom.

intraepithelial neoplasia (CIN) of grades I and II, and types 16 and 18 in CIN III and cervical carcinomas [Gissmann et al, 1984].

A role for HPV in the progression of dysplastic lesions to malignancy still needs clarification, although it seems likely that many cofactors are involved. Epidemiological evidence shows sexual promiscuity, early onset of sexual activity, and smoking to be major risk factors, but the immune status and responsiveness of the individual may also be important.

It was recognised many years ago that renal transplant patients treated with immunosuppressive drugs often suffered from disseminated nonregressing warts [Spencer and Andersen, 1970] and that patients with deficiencies in cell-mediated immunity (CMI) secondary to Hodgkin's disease and chronic lymphatic leukaemia, were more prone to papilloma infections [Morison, 1975]. Furthermore, not only is the susceptibility to infection with different types of HPV related to the extent of CMI deficiency [Obalek et al, 1980], but the ability of papilloma viruses themselves to induce an impairment of nonspecific CMI is dependent on the viral type [Jablonska et al, 1982]. Using an in vitro assay of mitogen-induced lymphocyte transformation in patients with recalcitrant genital warts, Seski et al [1978] found that CMI was significantly reduced. However, a similar study of patients with CIN showed normal levels of responsiveness to three mitogens, except for a reduced response to phytohaemagglutinin in patients with carcinoma-in-situ [Neill, 1984].

Several in vitro assays of specific CMI to HPV have been tried in patients with warts at various sites. Using a leucocyte migration inhibition test, Morison [1975] showed that few people were positive while they had skin warts, but many were positive just after resolution. Similarly, using lymphocyte proliferation assays (LPA), stimulation was greatest in those who had warts in the recent past [Ivanyi and Morison, 1976; Lee and Eisinger, 1976]. Weak or negative responses were obtained in those patients who had warts of long duration.

Many studies of humoral immunity to HPV have been carried out on a variety of patients with skin and anogenital warts [Kienzler, 1985]. Early studies showed IgM antibodies present at the time of resolution, whereas IgG antibodies took many months to develop. The prevalence and titres were highest in regressing warts and lowest or nonexistent in warts of longest duration, greatest tumour burden, or lowest virion content. Antibody can persist for many years and reinfection can occur despite its presence [Cubie, 1972].

Pfister and Zur Hausen [1978] described a sensitive radioimmunoassay for specific HPV types and found HPV-1 antibodies in 50% of children, whereas HPV-4 antibodies were more prevalent in an older age group. They noted that 46% of patients with condylomata acuminata and 57% of patients with genital cancers had antibodies to HPV-1.

Raising antisera against purified papilloma virions reveals little antigenic cross-reactivity between different types, suggesting that the major capsid protein is type-specific [Orth and Favre, 1985]. However, antisera against alkali-disrupted or detergent-dissociated particles show that common antigenic determinants exist within the virus [Jenson et al, 1980]. In 1983, Baird used detergent-disrupted bovine papillomaviruses (BPV) as antigen in an ELISA test to study the humoral response in patients with CIN, invasive carcinoma of the cervix, and anogenital warts. All three groups of patients gave higher absorbancies than did control groups of children or adults. He also reported that

those with invasive carcinomas had higher mean titres than did the other two groups of patients.

It was felt that the systemic immune response to cervical HPV infection in association with CIN required clarification. In this study we have used LPA and ELISA as indicators of cellular and humoral immunity, with purified or detergent-disrupted HPV-1, HPV-2, and BPV-1 as antigens.

MATERIALS AND METHODS

Patients

Ten to 20 mls of venous blood were obtained from patients attending the Lothian Area Colposcopy Clinic and collected in preservative-free heparin. Patients were asked if they had warts at any site or had had warts or verrucas in the past. A histological report was obtained of cervical biopsies taken at the same time as the blood.

Mononuclear cells were purified by centrifugation in lymphopaque (Nyegaard, Oslo) and plasma collected for use in both the LPA and ELISA tests.

Preparation of Papilloma Virus Antigens

Parings from plantar warts clinically identified as myrmecia (HPV-1) and mosaic (HPV-2) were collected separately into phosphate-buffered saline (PBS) containing 200 units/ml penicillin and 200 μg/ml streptomycin. Pooled parings were thoroughly minced and ground with sterile sand in a mortar and virus extracted by the method of Gissman and Zur Hausen [1976]. Two cycles of purification through caesium chloride of density 1.33 were performed and the lower visible band containing full virion particles was collected and dialysed overnight against several changes of buffer. Analysis of viral DNA by restriction endonuclease digestion showed the two preparations to contain only type 1 or type 2 virions. The virus preparations were filter sterilised and stored in aliquots at -70°C. Purified bovine papilloma virus from a cheek fibropapilloma (BPV-1) was similarly prepared.

The purified antigens were used directly in some assays. In others they were treated by mixing 1:1 with 2% sodium dodecyl sulphate (SDS) and 2% mercaptoethanol (ME) in PBS, followed by ultrasonication for 1 minute. Occasionally in the LPA, an unpurified antigen was used that consisted of virions pelleted after grinding and sterilised by filtration. Further antigen preparations were prepared by extraction of pelleted virions with 0.1 M glycine buffer pH 9.0 [Booth et al, 1979].

Protein concentrations of all antigen preparations were measured by the Lowrey method and ranged from 0.2 to 1.0 mg/ml.

Lymphocyte Proliferation Assay (LPA)

Peripheral blood mononuclear cells were washed thoroughly three times with PBS containing 0.2% preservative-free heparin. The cells were resuspended in RPMI-1640 (Northumbria Biologicals Ltd, Cramlington, Northumberland, UK) with 15% autologous plasma at a concentration of 10^6 cells/ml. Antigen was added at a concentration of 0.04–25 μg/ml where appropriate; 200 microlitre volumes were dispensed into the wells of flat-bottomed microtitre plates (Falcon), five wells being set up for each antigen. The plates were incubated in a humidified atmosphere of 5% CO_2 for 8 days. For the final 24 hours, 0.75 μC_i ^{3}H-methyl thymidine (Amersham International, Little Chalfont, UK) was

added to each well. The cells were harvested using an Ilacon II harvester onto Titertek filter paper discs, which were counted in a toluene-based liquid scintillator for 1 minute. Lymphocyte proliferation was expressed as the stimulation index:

$$\text{S.I.} = \frac{\text{mean counts/min in the presence of antigen}}{\text{mean counts/min without antigen}}$$

An S.I. of 2.0 or greater was considered positive.

Enzyme-Linked Immunosorbent Assay (ELISA)

The assay was a modification of the micromethod originally described by Voller and Bidwell [1976]. Briefly, SDS.ME disrupted virions were diluted in carbonate coating buffer, pH 9.6 to 1–2 μg/ml; 100 μls were dispensed into alternate wells and PBS + 1% bovine serum albumin (BSA) was added to the remaining wells as a background control. After overnight incubation at 4°C and washing four times with buffer (PBS + 0.05% Tween 20 + 0.1% BSA), 100 μl of PBS + 3% BSA was added for 1 hour at room temperature to block the remaining combining sites on the plastic [Forghani and Schmidt, 1979]. Washed plates were dried and could be stored for up to 10 days at 4°C before use. Thereafter, plasma (1:50) and antihuman alkaline phosphatase (1:500; Sigma Chemical Co., St. Louis, MO) diluted in PBS + 1% BSA were added for 2½ hours and 2 hours, respectively, both at 37°C. Substrate (paranitrophenyl phosphate, 1 mg/ml in freshly prepared diethanolamine buffer pH 9.8 without $MgCl_2$) was added for 20–30 minutes before stopping with 3M NaOH. The absorbance of each sample was measured spectrophotometrically. In each run a positive control (from a healthy staff member with a high titre to HPV-1 and HPV-2) and at least three negative controls were included. The negatives were selected from colposcopy patients with no known history of warts, no koilocytes, or warty atypia in histological sections and who repeatedly gave very low absorbancies on ELISA screening.

The corrected absorbance was calculated for each sample by subtracting the absorbance of the background control well from that of the antigen-containing well. The ELISA Index (E.I.) was calculated by dividing the absorbancy of the test sample by the mean absorbance of at least three negative samples plus three standard deviations.

RESULTS

The lymphocyte proliferation assay is a useful in vitro guide to the existence of T-cells already sensitised to a particular antigen in vivo. It was of interest to ascertain if proliferation occurred in response to papilloma virus antigens when lymphocytes from patients with CIN were used. Of the 92 such patients studied, 23 (25%) gave a positive stimulation index (S.I. > 2.0) with at least one of the antigens chosen and at one or more concentrations of that antigen. As may be seen from Table I, positive responses were obtained in patients with all grades of CIN and there was no significant difference between the number in each group. Of the 23 positive responses, 14 were from patients with histological evidence of koilocytes and 9 from patients without koilocytes. The blastogenic response to the antigen was low as reflected in the stimulation indices obtained (Fig. 1). Figure 1 includes several different antigenic preparations used for each virus and, therefore, gives the number of patient tests performed rather than the number of patients

TABLE I. Response of Peripheral Blood Lymphocytes to Papilloma Antigens in Patients With Various Degrees of CIN as Shown by LPA

Grade of dysplasia	No. of patients	No. with koilocytes (K+) or without (K−)	No. of patients with or without koilocytes giving S.I. > 2.0	Total no. with S.I. > 2.0
No dysplasia at time of assay	28	13 K+ (past) 15 K− (past)	3 K+ 2 K−	5 (17.9%)
CIN I	14	7 K+ 7 K−	3 K+ 1 K−	4 (28.5%)
CIN II	22	14 K+ 8 K−	6 K+ 3 K−	9 (40.9%)
CIN III	28	11 K+ 17 K−	2 K+ 3 K−	5 (17.4%)
Totals	92	45 K+ 47 K−	14 K+ (31.1%) 9 K− (19.1%)	23 (25%)

themselves. Only the optimum concentration of a particular preparation for each patient is given.

More positive results were obtained with the purified antigens (19/63; 30.2% of those tested) than with SDS/ME treatment (6/28; 21.4%) or using unpurified virions (3/16; 18.8%). Fewest positive results were obtained with the glycine extract (2/26; 7.7%). Nevertheless, several patients reacted only to one or other disrupted antigens and not to the purified preparations.

Whereas no correlation with koilocytosis was found, the positive cases came almost exclusively from those with a history of skin warts (Table II). Out of 68 of those who knew

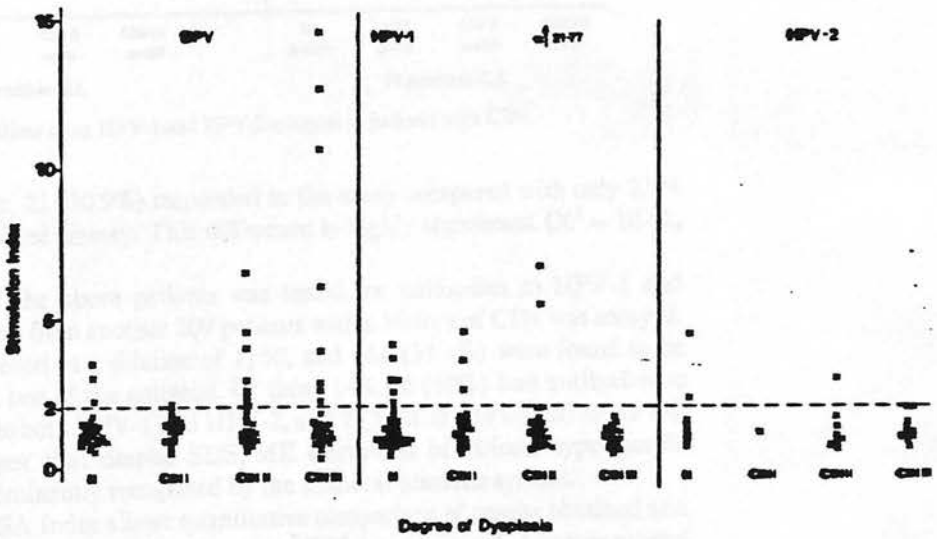


Fig. 1. Stimulation indices in LPA using BPV-1, HPV-1 and HPV-2 antigens in patients with CIN.

TABLE II. Influence of Skin Wart History on the Outcome of LPA Against Papilloma Antigens in Patients With CIN

Skin wart history	No. of patients	No. with S.I. > 2.0	Presence (K+) or absence (K−) of koilocytes in positive responders	
Past or present warts	68	21 (30.9%)	13 K+	8 K−
No known history	24	2 (8.3%)	1 K+	1 K−
Totals	92	23	14 K+	9 K−

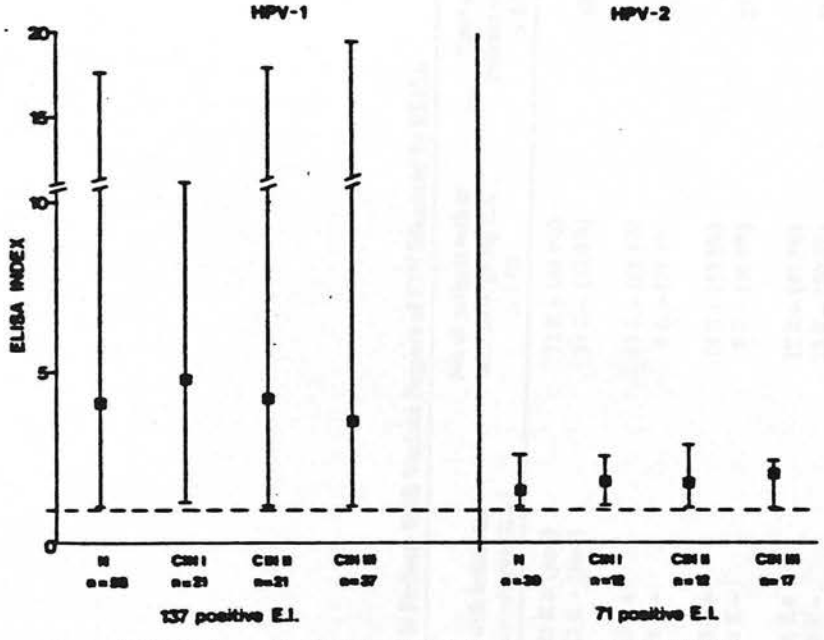


Fig. 2. ELISA indices using HPV-1 and HPV-2 antigens in patients with CIN.

they had or had had warts, 21 (30.9%) responded in the assay compared with only 2/24 (8.3%) with no acknowledged history. This difference is highly significant ($X^2 = 10.42$, $p = 0.005$).

Plasma from 73 of the above patients was tested for antibodies to HPV-1 and HPV-2. In addition, plasma from another 209 patients with a history of CIN was assayed. The specimens were screened at a dilution of 1/50, and 144 (51.1%) were found to be screen-positive to at least one of the antigens. Of those 144, 72 (50%) had antibodies to HPV-1 only, 65 (45.1%) to both HPV-1 and HPV-2, and 7 (5.8% of 139 tested) to HPV-2 only. These results suggest that despite SDS/ME disruption of virions, type-specific epitopes were being predominantly recognised by the humoral immune system.

The use of the ELISA Index allows quantitative comparison of results obtained at a single screening dilution to be made. With disrupted HPV-1 as antigen, E.I. values ranged from 1 to nearly 20, but with disrupted HPV-2 as antigen, the range was restricted to less than 3 (Fig. 2).

No correlation between the presence of HPV antibodies and koilocytosis or degree of CIN was found (Table III). Indeed, the distribution of positive results between those with

TABLE III. Antibody Response to HPV-1 and HPV-2 in Patients With Various Degrees of CIN Measured by ELISA

Grade of dysplasia	No. of patients	No. with koilocytes (K+) or without (K−)	No. of patients with or without K giving E.I. > 1.00	Total no. of patients with E.I. > 1.00	Positive responders to different HPV types
No dysplasia at time of assay	117	45 K+ (past) 72 K− (past)	22 K+ (48.9%) 38 K− (52.8%)	60	30 Type 1 only 2 Type 2 only 28 Both types
CIN I	39	23 K+ 16 K−	15 K+ (65.2%) 6 K− (37.5%)	21	9 Type 1 only 0 Type 2 only 12 Both types
CIN II	42	26 K+ 16 K−	14 K+ (53.8%) 8 K− (50.0%)	22	10 Type 1 only 1 Type 2 only 11 Both types
CIN III	84	36 K+ 48 K−	17 K+ (47.2%) 24 K− (50.0%)	41	24 Type 1 only 4 Type 2 only 13 Both types
Totals	282	130 K+ 152 K−	68 K+ (52.3%) 76 K− (50.0%)	144	73 Type 1 only 7 Type 2 only 64 Both types

TABLE IV. Influence of Skin Wart History on Antibody Response to HPV-1 and HPV-2 in Patients With CIN

Skin wart history	No. of Patients	No. with E.I. > 1.00 to HPV-1 only	HVP-2 only	Both HPV 1 & 2	Total no. positives	Presence (K+) or absence (K−) of koilocytes in positive responders	
Past or present	196	50	5	47	102 (52.0%)	51 K+	51 K−
No known history	86	23	2	17	42 (48.8%)	17 K+	25 K−
Totals	282	73	7	64	144 (51.1%)	68 K+	76 K−

and without koilocytosis and in all grades of dysplasia is remarkably consistent. This finding mirrors the results obtained in the LPA. Table IV shows the distribution of antibody-positive patients between the two groups: those with a past or present history of skin warts and those with no known history of skin warts. Whereas 102/144 (70.8%) of the positive ELISA results came from patients who knew they had had skin warts, the proportions within the two groups were similar.

Apart from the greater sensitivity of the ELISA (51.1% positivity compared with 25% by LPA), both assays gave essentially similar results. Nevertheless, there was little correlation between the ELISA and LPS results in the same patients. Of the 18 positive LPA specimens, 8 had no evidence of antibodies; of the 38 positive by ELISA, only 10 reacted also in LPA. A further 27 of the 73 tested were negative in both tests. Thus, in patients with CIN, the response, both cellular and humoral, to antigens derived from HPV-1 and HPV-2 reflects exposure to those types of papilloma virus and does not correlate with koilocytosis or grade of CIN.

DISCUSSION

In the LPA, 25% of the 92 patients gave a stimulation index greater than two against at least one papilloma antigen preparation. Lymphocytes from various patients responded differently to different preparations: thus, one patient had an S.I. of 12.7 using CsCl-purified BPV as antigen and 14.7 using SDS.ME-disrupted BPV, whereas another gave an S.I. of 1.26 with CsCl purified BPV and 10.7 with SDS.ME disrupted BPV. Perhaps it is surprising that any patient should react to purified virions of BPV, although Lancaster and Olson [1982] showed that 2/10 milk samples contained sufficient BPV particles to be detectable microscopically. SDS is toxic to lymphocytes, and in many patients the use of SDS.ME disrupted antigen led to cell death, but occasionally, as in the two patients described above, dilution of the antigenic preparation from 100- to 10,000-fold eliminated this problem.

LPAs for viral antigens usually require 5 or 6 days to obtain measurable responses by incorporation of ^{3}H-thymidine. Lee and Eisinger [1976] used 6 days in their papilloma virus assay but reported that the blastogenic response was low at this time. An 8-day assay was found to yield more positive responses but led to larger standard errors (generally in the range of 10–25% between quintuplicate wells). The consistency of the response could not be significantly improved by feeding the cultures with additional medium at 5 days, nor by adding interleukin-2 either on day 0 or day 4. It was felt that in many cases the

variation observed reflected a borderline response possibly due to a limited number of T-memory cells in peripheral blood.

More patients with koilocytes gave a positive response in the LPA than did those without when all grades of dysplasia were taken together (Table I), but the difference is not significant. More patients with CIN II gave S.I. greater than two than patients in other groups, but again the difference is not significant. On the other hand, 21/23 positive patients gave a history of skin warts, mainly on hands and feet. This association is very significant and suggests that, despite disruption of the papilloma particles to reveal internal epitopes, a type-specific memory response to HPV-1 and HPV-2 infections is being detected.

The ELISA test is an increasingly used and sensitive assay adaptable for antigen or antibody detection. In this study it has been used to detect circulating IgG antibodies to SDS.ME disrupted HPV-1 and HPV-2 in patients with CIN. Just over 50% of patients were found to have detectable antibodies to at least one of these types. These results are comparable to those of Pfister and Zur Hausen [1978] already noted.

Disruption of virions by SDS.ME is thought to reveal a group-specific antigen to which animal antisera with cross-reactivity can be prepared [Jenson et al, 1980]. In vivo these internal common antigens may not be exposed to the immune system. That the ELISA test described here detects type-specific antigens is shown by the fact that some patients reacted with HPV-2 and not HPV-1 (Table IV). The 64 patients who had antibodies to both HPV-1 and HPV-2 have probably had infections caused by both viral types, although it is also possible that some effect of cross-reactivity due to the use of disrupted particle is being seen. No cross-reactivity, however, was apparent with disrupted BPV: 55 patients gave negligible absorbancies (<0.13) with disrupted BPV used at similar concentrations to the HPV-1 and HPV-2 antigens in ELISA where absorbancies with HPV-1 ranged from 0.23 to 1.21 and with HPV-2 from 0.24 to 0.56. These variations in absorbancies (and thus in E.I.) may reflect differences in exposure to antigen. In HPV-1 lesions, there is greater antigen production and virion release than in HPV-2 lesions. The exposure to BPV will be much less. It is questionable, therefore, if the small differences in absorbancies noted by Baird [1983] in patients with CIN, genital warts, and cervical carcinoma are significant.

The distribution of antibody to HPV-1 and HPV-2 (Table III) and its extent (measured by E.I., Fig. 2) are fairly evenly matched across all grades of CIN, whether or not koilocytes are present. This suggests a response to an HPV other than that producing the cervical lesion, and indeed supports the concept of a type-specific reaction to a skin wart infection.

Ivanyi and Morison [1976] noted no correlation between positive lymphocyte migration inhibition responses and the presence of antibody. Similarly in this study, there was little correlation between LPA and ELISA results, with 36/73 samples giving positive responses in only one test. In an investigation of CMI to BK virus, a member of the polyoma subgroup of papovaviruses, Drummond et al [1985] noted no correlation between lymphoproliferation and antibody, also using LPA and ELISA.

It is apparent from these results that further study of the cellular and humoral responses to papilloma virus in patients with cervical lesions requires the use of type-specific antigens. However, virus production in genital papillomas and CIN is too low to prepare sufficient amounts of viral antigen necessary to establish satisfactory serological tests [Gissman and Gross, 1985]. Nevertheless, evidence is accumulating that early

proteins, particularly those coded by the E6 and E7 open reading frames (ORFs) of the papilloma genone, can be produced both in a cervical carcinoma [Smotkin and Wettstein, 1986] and in cultured cervical carcinoma cells [Seedorf et al, 1987]. Fusion proteins, produced by the transcription of fragments of papilloma virus DNA containing the appropriate ORFs attached to an expression vector in competent bacterial cells, have already been made to the E4 ORF of HPV-1 [Doorbar et al, 1986]. Fusion proteins to the early genes of the HPV types involved in genital lesions could be similarly produced and used in both the ELISA and LPA. Such work is already in progress (McCance, personal communication).

Some of the early work on HPV antigens and antibodies suggested that antibodies were produced against wart virus infected cells [Matthews and Shirodaria, 1973]. This requires further investigation, and the extraction of such antigens from cervical biopsy material and their use in assays such as those described above could provide another approach for the study of the immune responses in patients with papilloma virus associated cervical lesions.

ACKNOWLEDGMENTS

We would like to thank Dr. G.E. Smart, Dr. S. Fletcher, and the staff of the Lothian Area Colposcopy Clinic for providing access to their patients and for their help in providing clinical and histological information; Mrs. M. Murray for collecting the blood samples and wart histories; and Dr. B. Barr for analysis of the viral DNA type of the antigen preparations used. We thank the Cancer Research Campaign for funding this project.

REFERENCES

Baird PJ (1983): Serological evidence for the association of papilloma virus and cervical neoplasia. Lancet ii:17–18.

Booth JC, Harrington G, Aziz TA, Stern H (1979): Comparison of enzyme linked immunosorbent assay (ELISA) technique and complement fixation test for estimation of cytomegalovirus IgG antibody. Journal of Clinical Pathology 32:122–127.

Cubie HA (1972): Serological studies in a student population prone to infection with human papilloma virus. Journal of Hygiene (Camb.) 70:677–690.

Doorbar J, Campbell D, Grand RJA, Gallimore PH (1986): Identification of the human papilloma virus-1a E4 gene products. The EMBO Journal 5:355–362.

Drummond JE, Shah KV, Donnenberg AD (1985): Cell-mediated immune responses to BK virus in normal individuals. Journal of Medical Virology 17:237–247.

Forghani B, Schmidt N (1979): Antigen requirements, sensitivity and specificity of enzyme immunoassays for measles and rubella viral antibodies. Journal of Clinical Microbiology 9:657–664.

Gissman L, Zur Hausen H (1976): Human papilloma virus DNA: physical mapping and genetic heterogeneity. Proceedings of the National Academy of Sciences USA 73:1310–1313.

Gissmann L, Gross G (1985): Association of HPV with human genital tumours. Clinics in Dermatology 3:124–129.

Gissmann L, Boshart M, Durst M, Ikenberg H, Wagner D, Zur Hausen H (1984): Presence of human papillomavirus in genital tumours. Journal of Investigative Dermatology 83:26S–28S.

Ivanyi L, Morison WL (1976): *In vitro* lymphocyte stimulation by wart antigen in man. British Journal of Dermatology 94:523–527.

Jablonska S, Orth G, Lutzner MA (1982): Immunopathology of papillomavirus-induced tumours in different tissues. Springer Seminars in Immunopathology 5:33–62.

Jenson AB, Rosenthal JR, Olson C, Pass F, Lancaster WD, Shah K (1980): Immunological relatedness of papillomaviruses from different species. Journal of the National Cancer Institute 64:495–500.
Kienzler J-L (1985): Humoral immunity to human papillomaviruses. Clinics in Dermatology 3:144–156.
Koss LG, Durfee GR (1956): Unusual patterns of squamous epithelium of the uterine cervix: Cytologic and pathologic study of koilocytic atypia. Annals of the New York Academy of Science 63:1245–1261.
Lancaster WD, Olson C (1982): Animal Papillomaviruses. Microbiological Reviews 46:191–207.
Lee AKY, Eisinger M (1976): Cell-mediated immunity (CMI) to human wart virus and wart-associated tissue antigens. Clinical and Experimental Immunology 26:419–424.
Matthews RS, Shirodaria PV (1973): Study of regressing warts by immunofluorescence. Lancet i:689–691.
Meisels A, Fortin R, Roy M (1977): Condylomatous lesion of the cervix II. Cytologic and histopathologic study. Acta Cytologica 21:379–390.
Morison WL (1975): Viral warts, herpes simplex and herpes zoster in patients with secondary immune deficiencies and neoplasms. British Journal of Dermatology 92:625–630.
Neill WA (1984): Cell mediated immune responses to cytomegalovirus in patients with abnormalities of the uterine cervix. M. Phil thesis, University of Edinburgh.
Obalek S, Glinski W, Hafleck M, Orth G, Jablonska S (1980): Comparative studies on cell-mediated immunity in patients with different warts. Dermatologica 161:73–83.
Orth G, Favre M (1985): Human papillomaviruses. Biochemical and Biological Properties. Clinics in Dermatology 3:27–42
Pfister H, Zur Hausen H (1978): Seroepidemiological studies of human papilloma virus (HPV-1) infections. International Journal of Cancer 21:161–165.
Seedorf K, Olkersdorf T, Krammer G, Rowekamp W (1987): Identification of early proteins of the human papilloma viruses type 16 (HPV-16) and type 18 (HPV-18) in cervical carcinoma cells. The EMBO Journal 6:139–144.
Seski JC, Reinhalter ER, Silva J (1978): Abnormalities of lymphocyte transformation in women with condylomata acuminata. Obstetrics and Gynaecology 51:188–192.
Smotkin D, Wettstein FO (1986): Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proceedings of the National Academy of Sciences USA, 83:4680–4684.
Spencer ES, Andersen HK (1970): Clinically evident, non-terminal infections with herpesvirus and the wart virus in immunosuppressed renal allograft recipients. British Medical Journal iii:251–254.
Voller A, Bidwell DE (1975): A simple method for detecting antibodies to rubella. British Journal of Experimental Pathology, 56,338–340.

Journal of Virological Methods, 20 (1988) 239–249
Elsevier

JVM 00732

Synthetic oligonucleotide probes for the detection of human papilloma viruses by in situ hybridisation

Heather A. Cubie and Mary Norval

Department of Bacteriology, University of Edinburgh Medical School, Edinburgh EH8 9AG, U.K.

(Accepted 10 March 1988)

Summary

Using the published nucleotide sequence data for human papilloma virus (HPV) types 1, 6 and 16, sequences of 30 bases in length from the beginning of the E6 open reading frame (ORF) were selected. Oligonucleotides were synthesised on an Oswel Gene Synthesiser and labelled at the 3′ end with biotin using the enzyme terminal transferase. In situ hybridisation was carried out on paraffin sections of wart and cervical tissues mounted on silanated slides. A 2 h hybridisation step allowed the whole process to be completed within one working day.

The technique successfully demonstrated the presence of HPV-1 in skin warts, and of HPV-6 and HPV-16 in genital warts and cervical lesions. This simple approach has diagnostic potential for the detection and typing of papilloma viruses in biopsy material.

Oligonucleotide; Human papilloma virus; In situ hybridization

Introduction

Nucleic acid hybridisation is becoming an increasingly used tool in virology (for review see Norval and Bingham, 1987), either by Southern blotting, dot-blots or in situ hybridisation. However, the full potential of hybridisation has yet to be realised for the diagnosis of viral diseases. Current methods often employ expensive

Correspondence to: Heather A. Cubie, Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

radiolabelled probes with limited half-lives and lengthy assay procedures. Brigati et al. (1983) first reported the use of biotin-labelled probes for the detection of different viruses in infected cells. Since then rapid in situ hybridisation techniques with biotinylated probes for herpes simplex virus (Forghani et al., 1985) and for cytomegalovirus (Ungar et al., 1986) have been developed.

Most methods rely on probes prepared from cloned nucleic acid but few diagnostic virology laboratories have the expertise, large scale bacterial culture systems and containment facilities required for such production. Ten years ago Brahic and Haase (1978) suggested that DNAse digestion to reduce probe size to approximately 50 nucleotides would increase diffusion and therefore the efficiency of reaction three-fold. Very recently, Lin et al. (1987) reported the use of a customised 21-nucleotide sequence (21-mer) probe, end-labelled with ^{32}P, for the detection of hepatitis B virus DNA in serum by dot-blot hybridisation and found that it gave an equally sensitive but quicker and simpler assay than that using ^{32}P-labelled by nick translation genomic probes. Similarly, Robertson et al. (1987) used a 50-mer probe complementary to the measles virus nucleocapsid gene to identify persistent measles in lymphocytes from patients with autoimmune chronic active hepatitis.

For human papilloma virus (HPV), DNA hybridisation is currently the only way to distinguish different virus types. Radiolabelled DNA probes have been generally favoured in this situation because of their acknowledged greater sensitivity over non-radiolabelled probes (Burns et al., 1987). HPV DNA in cells obtained from cervical smears or scrapes has been successfully detected with ^{32}P-labelled type-specific probes in dot-blot assays (Wagner et al., 1984; Wickenden et al., 1985). However, histology is a useful adjunct in diagnosing HPV infection in areas such as the cervix where epithelial dysplasia is commonly associated with viral infection. In studies using in situ hybridisation to reveal the HPV types involved in cervical intra-epithelial neoplasia (CIN), radiolabelled probes have been used (Gupta et al., 1985; Syrjanen et al., 1986). Several attempts have been made to amplify the detection of bound biotinylated HPV probes, for example by introducing additional biotin-avidin layers (Beckmann et al., 1985) or by silver enhancement of horseradish peroxidase staining in a method capable of detecting as few as 10 copies of HPV DNA per cell (Burns et al., 1987).

We have tried a similar approach to that described by Lin et al. (1987), using synthetically produced 30-mers with sequences obtained from the published nucleotide sequence data for HPV types 1, 6 and 16. These were end-labelled with biotin and tested on paraffin sections of tissue by in situ hybridisation.

Materials and Methods

Biopsy material

Sections of formalin-fixed paraffin-embedded tissue from skin wart lesions, genital lesions and areas of cervical dysplasia were obtained from the Department of Pathology, University of Edinburgh. The sections were floated onto silanated slides

prepared according to the method described by Tourtellotte et al. (1987). Slides were used within 8 weeks of coating. A few slides were treated by dipping in 2% aminopropyltriethoxysilane (Tespa™; Sigma, Poole) in acetone as described by Burns et al. (1987).

Synthetic oligonucleotides

Published sequence data for HPV-1a (Danos et al., 1982), HPV-6b (Schwartz et al., 1983) and HPV-16 (Seedorf et al., 1985) were studied and the E6 nucleotide sequences compared. Alignment of the sequences to allow comparison of the E6 proteins was described by Giri and Danos (1986) and the 30 nucleotides which occur immediately downstream of the ATG start codon of E6 were selected and are shown in Fig. 1.

The entire GenBank database (12537 nucleotide sequence files) was searched for homologies to the chosen oligonucleotide sequences using the University of Wisconsin Genetics Computer Group software (Devereux et al., 1984). Variable levels of mismatching were permitted, mismatches of 9 or more bases being assumed to be insignificant. Only two comparable sequences were found. These were (i) HPV-11 differing from HPV-6 in 4 bases and (ii) a sequence from within the macrophage Fc-gamma R-beta 2 cDNA which had seven mismatches when compared with the sequence selected for HPV-16.

The chosen 30-mer sequences were synthesised on an Oswel Gene Synthesiser in the Department of Chemistry, University of Edinburgh, using B-cyanoethyl phosphoramidite chemistry. The DNA was produced as 2 μmol contained in 1 ml of distilled water and had −OH 5′ and 3′ termini. It was purified by reverse phase HPLC and subsequently desalted by gel filtration.

	5' Base	107								136
HPV-1a	(ATG)	GCG	ACA	CCA	ATC	CGG	ACC	GTC	AGA	CAG	CTT
		105								134
HPV-6b	(ATG)	GAA	AGT	GCA	AAT	GCC	TCC	ACG	TCT	GCA	ACG
		105								134
HPV-11	(ATG)	GAA	AGT	AAA	GAT	GCC	TCC	ACG	TCT	GCA	ACA
		107								136
HPV-16	(ATG)	TTT	CAG	GAC	CCA	CAG	GAG	CGA	CCC	AGA	AAG

Fig. 1. Oligonucleotide sequences of 30 bases in length selected for HPV-1a, HPV-6b and HPV-16, and including HPV-11 for comparison.

Labelling of oligonucleotides

The oligonucleotides were labelled at the 3′ end using the enzyme terminal transferase (Amersham, U.K.) and a biotinylated nucleotide, biotin-11-dUTP (BRL Ltd., Paisley). The reagents were mixed in the proportions 3 μl of 30-mer in 12 μl distilled water, with 5 μl sodium cacodylate buffer and 10 μl enzyme (Amersham 3′ end-labelling kit) and 25 μl biotin-11-dUTP (BRL Ltd.). The reagents were carefully mixed, incubated at 37°C for 2 h and the reaction stopped by loading directly onto a pre-packed column containing Sephadex G-50 DNA grade (Nick™ -column; Pharmacia Ltd., Milton Keynes) and eluted with TE buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl) containing 0.1% SDS. Four drop fractions were collected and 2 μl of each were spotted onto nitrocellulose membranes, dried and vacuum baked for 30 min at 80°C. The labelled probe was detected with streptavidin, biotin-alkaline phosphatase and nitroblue/tetrazolium bromochloroindolyl phosphate (NBT/BCIP) as substrate (BRL DNA detection system) according to the manufacturer's instructions. Strongly positive fractions were pooled and stored in aliquots at −70°C.

In situ hybridisation with oligonucleotide probes

The paraffin sections were dewaxed in xylene twice for 10 min, washed in absolute alcohol twice for 10 min and rehydrated to water. Target DNA was unmasked by treatment with 0.2 M HCl for 10 min followed by a 5 min wash in distilled water and exposure to 0.2 mg/ml proteinase K (Sigma) in 50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA for 15 min at room temperature followed by two 5 min washes in Tris-buffered saline (5 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.2% glycine. The sections were dehydrated and air dried.

The probe was added in the proportion 1:10 to the high stringency buffer described by Ungar et al., (1986). Sections were overlaid with 20–50 μl of this mixture and covered with a plastic coverslip cut from an autoclavable polypropylene bag, placed on a metal tray and floated on a water bath at 90°C for 10 min. The tray was transferred briefly to a 37°C water bath and the slides placed in moist boxes at 42°C for 2 h.

After hybridisation the coverslips were removed by gentle agitation of the slides in 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0). Post-hybridisation washes were carried out by the method of Lewis et al. (1987), or more briefly by three 5 min washes in 2 × SSC.

Hybridised DNA was detected using the streptavidin-biotinylated alkaline phosphatase and NBT/BCIP detection system described above, except that incubation periods with streptavidin and biotin-AP were increased to 10 min. After colour development, generally between 1–2 h, the reaction was stopped with 20 mM Tris-HCl, pH 7.4, 5 mM EDTA and the slides mounted without counterstaining in glycerol-gelatin.

In situ hybridisation with cloned HPV probes

HPV 1a, 11 and 16 DNA inserted in pBr 322 were kindly provided by Dr. Harold Zur Hausen, Heidelberg, F.R.G., amplified in *Escherichia coli* K101 and the

DNA extracted by alkaline lysis and chloroform treatment (Maniatis et al., 1982). The HPV-DNA still attached to plasmid was labelled with biotin-11-dUTP by nick translation (BRL Nick Translation System; BRL Ltd.) and the labelled probe separated by fractionation on a Sephadex G50 column. The plasmid alone was similarly treated and labelled.

Paraffin sections were dewaxed and rehydrated and the target DNA unmasked by the method described by Lewis et al. (1987). This includes not only HCl and proteinase K treatment, but also brief exposures to Triton X-100 and acetic acid and post-fixation in 4% paraformaldehyde. Denaturation and hybridisation were carried out as for the oligonucleotide hybridisations, except that a hybridisation time of 16–18 h was employed. Post-hybridisation washes and DNA detection were as described above.

Results

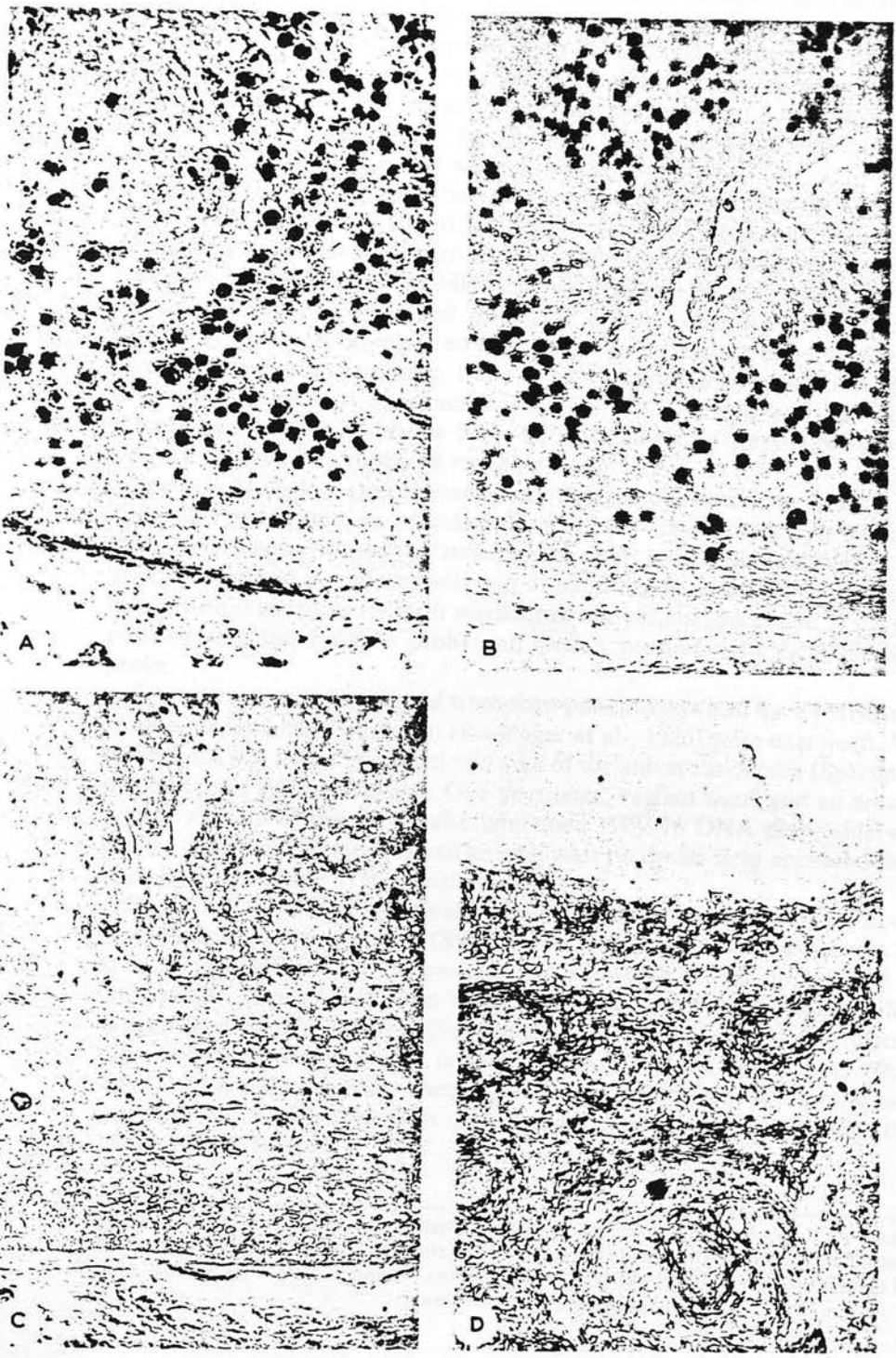
The loss of sections from glass slides was prevented totally by coating with organosilane according to the method described by Tourtellotte et al. (1987). It was

TABLE 1

In situ hybridisation results using genomic and synthetic oligonucleotide probes of HPV DNA types on selected tissue specimens

Specimen No.	Histological appearance	HPV–DNA probe					
		1	11	16	1E6	6E6	16E6
1	Hyperkeratotic hand wart	+++	–	–	+++	–	–
2	Simple plantar wart	++	–	–	++	–	–
3	Keratotic wart on forearm	–	–	–	–	–	–
4	Keratotic nodule on arm	–	–	–	+/–	–	–
5	Vulvar warts K+ no dysplasia	–	++	–	–	(+)	–
6	Perianal warts K+ severe dysplasia	ND	ND	ND	–	+	–
7	Anal wart same patient as No. 6, 1 yr later	ND	(+)	–	–	++	–
8	Vulvar carcinoma	ND	ND	–	–	ND	(+)
9	Vaginal warts, K+, no dysplasia, same patient as No. 8, 1 yr later	ND	–	+	–	–	+
10	Cervical lesion, CIN II, same patient as No. 9 at same time	ND	–	+	–	–	+
11	Anal wart, same patient as No. 8, 3 yr later	–	–	+	–	+	+
12	Cervical lesion, CIN II K+	ND	–	–	–	–	+
13	Cervical lesion, CIN II K+	–	–	+	–	(+)	+
14	Cervical lesion, CIN III K+	–	–	–	–	–	–
15	Cervical lesion, CIN II K+	–	–	+	–	–	+

K+ = koilocytes present; CIN II = moderate dysplasia; CIN III = severe dysplasia/carcinoma in situ. +++ = large numbers of nuclei containing HPV DNA; ++ = frequent patches of nuclei containing HPV DNA; + = infrequent patches of less well stained nuclei; (+) = HPV-containing nuclei only found with careful searching; +/– = clear nuclear staining found on one occasion but not on repeat testing.


A
B
C
D

necessary to stain the sections within 8 weeks, otherwise some detachment occurred. Silanation by dipping in 2% aminopropyl triethoxysilane in acetone was much quicker but an occasional section still detached during pre-treatment or hybridisation even on freshly coated slides. Sections of Specimen 2 (Table 1) were provided on uncoated slides and many of these were lost during processing. The concentration of proteinase K was found to be critical in maintaining the integrity of the sections and 0.2 mg/ml was optimal in our system.

Material from four skin warts was examined (Table 1). Two of these contained HPV-1 DNA as shown by strong hybridisation with both the HPV-1 probe ^{32}P-labelled by nick translation and the HPV-1E6 oligonucleotide probe (Fig. 2A,B). Neither genomic probes for HPV-11, HPV-16 and pBr 322 alone, nor oligonucleotide probes for HPV-6 and 16 (Fig. 2C) showed hybridisation. Consistent results were obtained on repeated testing with hybridisation down to the parabasal layer being noted in Specimen 1 and in many scattered positive nuclei throughout the cross-cut tissue of Specimen 2 (Fig. 2D). On a single occasion, Specimen 4 showed hybridisation with the HPV-1E6 oligoprobe in a restricted area but this was not observed on repeated retesting.

Sections from five genital warts were examined (Specimens 5, 6, 7, 9 and 11; Table 1). Two specimens of anal warts from the same patient hybridised with the HPV-6E6 oligoprobe but the staining was more intense in Specimen 7 (Fig. 3A) than in Specimen 6, and was only just discernible with the HPV-11 genomic probe in the former specimen. Vulvar warts from one patient (Specimen 5) were strongly positive with the HPV-11 probe and weakly positive with the HPV-6E6 oligoprobe.

Four specimens from a renal transplant patient known to have harboured HPV-16 in a malignant vulvar lesion (Rudlinger et al., 1986) were examined. Weak hybridisation was found in a restricted area of the vulvar carcinoma (Specimen 8) but only with the synthetic probe. One year later, vaginal warts and an area of moderate dysplasia of the cervix also contained HPV-16 DNA detectable with both probes (Specimens 9 and 10) and an anal wart two years later appeared to harbour both HPV-6 and HPV-16 (Specimen 11).

Finally, four cervical lesions showing koilocytosis and dysplasia of moderate to severe degree were examined (Specimens 12–15) and HPV-16 DNA was detected in 3. In two, HPV-16 DNA was detected with both the oligoprobe and the genomic probe. One of these also hybridised weakly with the HPV-6E6 oligoprobe suggesting a double infection (Specimen 13) (Fig. 3B). With both probes the positive cells were found mainly in the lower layers of the epithelium. While these small cells could be confused with infiltrating lymphocytes, no signs of inflammation were observed in this lesion. A further specimen hybridised only with the HPV-16E6 oligoprobe.

Fig. 2. In situ hybridisation in skin warts: sections of hyperkeratotic hand wart (Specimen 1) hybridised with: (A) biotin-labelled genomic HPV-1 DNA, (B) biotin-labelled HPV1-E6 oligoprobe, (C) biotin-labelled HPV-16E6 oligoprobe and (D) section of simple plantar wart (Specimen 2) hybridised with HPV1-E6 oligoprobe.

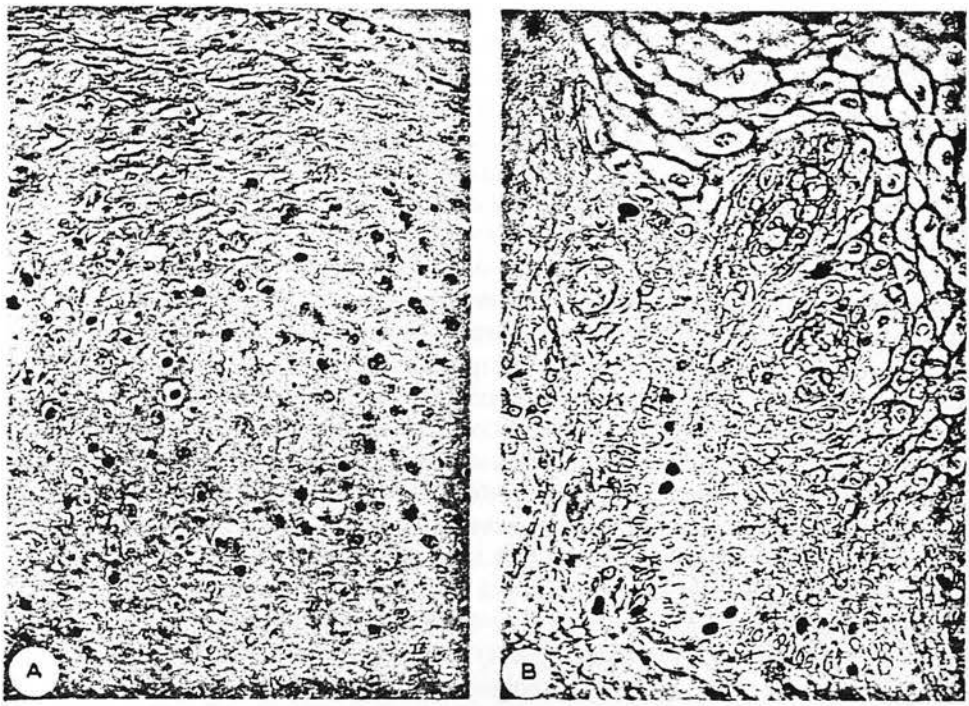


Fig. 3. (A)Section of an anal wart (Specimen 7) probed with HPV-6E6 oligoprobe. (B) Section of cervical lesion (Specimen 13) probed with HPV-16E6 oligoprobe.

Discussion

Paraffin embedded sections were used in this study because they are easy to obtain and retrospective analysis is possible. For a truly rapid typing service, cryostat sections would be preferable and work is under way to determine the optimal conditions for oligoprobes on frozen sections. Whether frozen or paraffin sections are used, in situ hybridisation enables the extent and site of infection to be seen. It has been suggested that non-invasive methods for obtaining cervical scrapes would be preferable to biopsy for typing of HPV (Wickenden et al., 1985; Webb et al., 1987), but it is interesting to note that positive areas of HPV hybridisation in genital lesions are often confined to the crevices (Chou et al., 1987) and might be missed by superficial scraping. A similar confinement of hybridisation to crevices in genital warts and to lower layers of epithelium in cervical lesions was noted in the current study.

Selecting the best oligonucleotide sequences to give type specific probes was difficult. Those human papillomaviruses whose complete DNA sequences have been determined show similar organisation with the conserved open reading frames being found in similar positions on a single strand of viral DNA. Sequence comparisons

also show a great degree of homology particularly in the L1 region which codes for the major structural antigen. It might have been possible to select a sequence from within L2 which is less conserved, to give type specific oligonucleotides, but it was decided that a sequence from within the E6/E7 region which is important for transformation might give wider variation between strains with varying degrees of oncogenic involvement (Giri and Danos, 1986).

Since HPV-6 and HPV-11 oligoprobes varied in only four bases it was thought likely that some cross-hybridisation between these types would occur. This was seen in Specimen 5 where a much stronger signal was obtained with the HPV-11 cloned probe than with the HPV-6E6 oligoprobe (Fig. 3) and conversely in Specimen 7 where HPV-6E6 staining was much more intense. Such limited cross-hybridisation suggests that the oligonucleotide probes are selective for individual HPV types. Lin et al. (1987) chose their oligoprobe to hepatitis B virus to be universal and it might be possible to have one universal probe for all HPV types possibly selected from the conserved L1 sequence, followed by type specific probes where appropriate.

Of great interest are the cases of CIN (Specimen 13) and of genital warts (Specimen 11) where positive signals, albeit weak, were detected with both HPV-6E6 and HPV-16E6. There is increasing evidence that infection with more than one HPV type occurs. McCance et al. (1985) described 5 women with multifocal genital intra-epithelial neoplasms in each of whom HPV-6 and 16 was found. Winkler et al. (1986) described HPV-6 and 16 in 65% of 43 patients with similar lesions, and recently Del Mistro et al. (1987) identified two types of HPV in 13 of 15 males with urethral condylomata acuminata.

Good agreement was obtained between hybridisation results using cloned HPV probes and oligoprobes. The saving in time, with the 2 h hybridisation step allowing completion of the process within one working day, makes the technique of in situ hybridisation with biotin-labelled synthetic oligonucleotides an attractive proposition when considering a diagnostic service, not only for HPV but for other viruses of clinical significance. The probes, free from contaminating vector sequences, can be produced in large quantities at reasonable cost and the use of totally defined sequences would allow better comparison of results obtained in different laboratories.

Acknowledgements

We would like to thank the following people in the University of Edinburgh for their help and support in this project: Dr. T. Brown, Department of Chemistry, for producing the synthetic oligonucleotides, Dr. R.W. Bingham, Department of Veterinary Pathology, for his GenBank searches, Dr. K. McLaren and Dr. S. Fletcher, Department of Pathology, for selecting the initial specimens and for helpful discussion over the analysis and to Dr. M. Bunney, Department of Dermatology and Dr G.E. Smart, Department of Gynaecology, for permission to study material from their patients.

The financial support of the Cancer Research Campaign is gratefully acknowledged.

References

Beckmann, A.M., Myerson, D., Daling, J.R., Kiviat, B.N., Fenoglio, C.M. and McDougall, J.K. (1985) Detection and localisation of human papillomavirus DNA in human genital condylomas by in situ hybridisation with biotinylated probes. J. Med. Virol. 16, 265–273.

Brahic, M. and Haase, A. (1978) Detection of viral sequences of low reiteration frequency by in situ hybridisation. Proc. Natl. Acad. Sci. USA 75, 6125–6129.

Brigati, D.J., Myerson, D., Leary, J.J., Spalholz, B., Travis, S.Z., Fong, C.K.Y., Hsiung, G.D. and Ward, D.C. (1983) Detection of viral genomes in cultured cells and paraffin embedded tissue sections using biotin-labelled hybridisation probes. Virology 126, 32–50.

Burns, J., Graham, A.K., Frank, C., Fleming, K.A., Evans, M.F. and McGee, J.O'D. (1987) Detection of low copy human papillomavirus DNA and mRNA in routine paraffin sections of cervix by non-isotopic in situ hybridisation. J. Clin. Pathol. 40, 858–864.

Chou, L.I., Hirochika, H., Nasseri, M., Stoler, M.H., Wolinsky, S.M., Chin, M.T., Hirochika, R., Arvan, D.S. and Broker, T.R. (1987) Cold Spring Harbour Laboratory. Cancer Cells 5, 55–72.

Danos, O., Katinka, M. and Yaniv, M. (1982) Human papillomavirus 1a complete DNA sequence: a novel type of genome organisation among papovaviridae. Eur. Mol. Biol. Organ. J. 1, 231–236.

Del Mistro, A., Braunstein, J.D., Halwer, M. and Koss, L.G. (1987) Identification of human papillomavirus types in male urethral condylomata acuminata by in situ hybridisation. Hum. Pathol. 18, 936–940.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12, 387–395.

Forghani, B., Dupuis, K.W. and Schmidt, N.J. (1985) Rapid detection of herpes simplex virus DNA in human brain tissue by in situ hybridisation. J. Clin. Microbiol. 22, 656–658.

Giri, I. and Danos, O. (1986) Papillomavirus genomes: from sequence data to biological properties. Trends Genet. 2, 227–232.

Gupta, J.W., Gendelman, H.E., Naghashfar, Z., Gupta, P., Rosenshein, N., Sawada, E., Woodruff, J.D. and Shah, K.V. (1985) Specific identification of human papillomavirus type in cervical smears and paraffin sections by in situ hybridisation with radioactive probes: a preliminary communication. Int. J. Gynecol. Pathol. 4, 211–218.

Lewis, F.A., Griffiths, S., Dunnicliff, R., Wells, M., Dudding, N. and Bird, C.C. (1987) Sensitive in situ hybridisation technique employing biotin-streptavidin-polyalkaline phosphatase complex. J. Clin. Pathol. 40, 163–166.

Lin, H.J., Wu, P.-C. and Lai, C.-L. (1987) An oligonucleotide probe for the detection of hepatitis B virus DNA in serum. J. Virol. Methods 15, 139–149.

McCance, D.J., Clarkson, P.K., Dyson, J.L., Walker, P.G. and Singer, A. (1985) Human papillomaviruses types 6 and 16: multifocal intraepithelial neoplasia of female lower genital tract. Br. J. Obstet. Gynaecol. 92, 1093–1100.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Norval, M. and Bingham, R.W. (1987) Advances in the use of nucleic acid probes in diagnosis of viral diseases of man. Brief review. Arch. Virol. 97, 151–165.

Robertson, D.A.F., Guy, E.C., Zhang, S.L. and Wright, R. (1987) Persistent measles genome in autoimmune chronic active hepatitis. Lancet ii, 9–11.

Rudlinger, R., Smith, I.W., Bunney, M.H. and Hunter, J.A.A. (1986) Human papillomavirus infections in a group of renal transplant recipients. Br. J. Dermatol. 115, 681–692.

Seedorf, K., Krammer, G., Durst, M., Suhai, S. and Rowekamp, W.G. (1985) Human papillomavirus type 16 DNA sequence. Virology 145, 181–185.

Syrjanen, S., Syrjanen, K., Mantyjarvi, R., Parkkinen, S., Vayrynen, M., Saarikoski, S. and Castren, O. (1986) Human papillomavirus (HPV) DNA sequences demonstrated by in situ DNA hybridisation in serial paraffin-embedded cervical biopsies. Arch. Gynecol. 239, 39–48.

Tourtellotte, W.N., Verity, A.N., Schmid, P., Martinez, S. and Shapshak, P. (1987) Covalent binding of formalin fixed paraffin embedded brain tissue sections to glass slides suitable for in situ hybridisation. J. Virol. Methods 15, 87–99.

Ungar, E.R., Budgeon, L.R., Myerson, D. and Brigati, D.J. (1986) Viral diagnosis by in situ hybridisation. Am. J. Surg. Pathol. 10, 1–8.

Wagner, D., Ikenberg, H., Boehm, N. and Gissmann, L. (1984) Identification of human papillomavirus in cervical swabs by deoxyribonucleic acid in situ hybridisation. Obstet. Gynecol. 64, 767–772.

Wickenden, C., Steele, A., Malcolm, A.D.B. and Coleman, D.V. (1985) Screening for wart virus infection in normal and abnormal crevices by DNA hybridisation of cervical scrapes. Lancet i. 65–67.

Winkler, B., Krumholz, B., Galli, R., Nizet, V., Palefsky, J., Schoolnik, G. and Steinberg, B. (1986) Multifocal lower female genital tract condyloma/neoplasia syndrome. J. Cell Biochem. Suppl. 10C, 221.